NOVEL METHODS OF DISEASE SURVEILLANCE IN WILDLIFE

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NOVEL METHODS OF DISEASE SURVEILLANCE IN WILDLIFE

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ABSTRACT

Both infectious and noninfectious disease agents in wildlife impact human health and accurate research, monitoring, and diagnostic methods are necessary. The objectives of the research reported here were to develop and implement novel methods for bacterial and toxicological disease agent surveillance in wildlife. This dissertation begins with a review of tularemia, an important zoonotic disease to the state of Alaska and the Northern hemisphere. In chapter two, I show the development and implementation of broad-based PCR and quantitative PCR (qPCR) surveillance methods for bacterial DNA in tissue samples; 1298 tissue samples were assayed, numerous potential bacterial pathogens were identified and qPCR detection limits were quantified for various tissue matrices. Chapter three describes an investigation into microbial infection as a source of embryo mortality in greater white-fronted geese (*Anser albifrons*) in Arctic Alaska. This chapter builds upon our previously developed PCR surveillance techniques by which I demonstrated that bacterial infection is responsible for some greater white-fronted goose embryo mortality in Arctic Alaska. Chapter four describes the development and validation of a cellulose filter paper method for quantifying total mercury in whole blood. I determined that filter paper technology is useful for monitoring total mercury in whole blood, with excellent recoveries (82 - 95% of expected values) and R^2 values (0.95 - 0.97) when regressed against the concentration of total mercury in whole blood, the technique generally considered as the "gold standard" for mercury detection. These methods will aid in the accurate detection of disease agents in wildlife as demonstrated by our white-fronted goose work.

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General Introduction

Human, animal, and ecosystem health are mutually interdependent and this relationship has been historically postulated on the basis of empirical observation. However, scientists, biologists, and human and animal health professionals often operate independently and may overlook interrelationships. To promote a collaborative effort between all those involved in medical care at large, in the early 2000's the One Health (OH) Initiative started taking shape. The mission statement of OH indicates that "One Health seeks to promote, improve, and defend the health and well-being of all species by enhancing cooperation and collaboration between physicians, veterinarians, other scientific health and environmental professionals and by promoting strengths in leadership and management to achieve these goals" (www.onehealthinitiative.com). The OH initiative is a collaborative effort between governmental agencies (e.g. the Centers for Disease Control and Prevention), nongovernmental organizations (e.g. the American Veterinary Medical Association), universities, and industry to promote the OH concept. Not only is the physical health of humans and animals related via disease, but humans' emotional well-being can be impacted by their perception of animal health, in that people feel good knowing that animal populations are healthy [1].

Alaska is a unique place to apply the OH concept. Many Alaska residents rely on wild animal resources for subsistence. Additionally, Alaska has a large tourism industry, much of which involves wildlife viewing. Alaska is also home to several zoonotic and wildlife diseases of importance [2]. Finally, climate change is resulting in warmer temperatures, which will theoretically cause drastic changes in host-pathogen interactions [3]. Therefore it is important that we monitor and study diseases in wild animal populations in Alaska.

In order to properly monitor animal diseases it is important understand these diseases and to have useful diagnostic tools. This dissertation begins by describing an example of a bacterial disease of importance to Alaska and the Northern hemisphere, tularemia. Then it describes broad-based polymerase chain reaction and sequencing methods for detection of bacterial DNA in wild animal tissues as a means of detecting and identifying disease agents. Next we applied these and other techniques to investigate avian embryo mortality in a field situation using those broad-based PCR and sequencing and other techniques. Finally, we move into the realm of noninfectious disease, and validate the use of a cellulose filter paper based sampling technique for use for mercury quantification in whole blood.

Tularemia is an example of a zoonotic bacterial disease that is classified as a category A select pathogen[1] by the CDC. Tularemia is caused by the Gram-negative bacterium *Francisella tularensis* [4]. *F. tularensis* is an obligate pathogen; rodents and lagomorphs are thought to be reservoir hosts [5], and is transmitted to humans either directly or via arthropod vectors. Depending on the subspecies of tularemia contracted and the route of entry, the disease can be up to 50% fatal in humans [6]. The pathological manifestations caused by *F. tularensis* may vary as a function of the route of entry and can present itself as infection of skin and lymph nodes (ulceroglandular), or lungs (pneumonic, the most serious form) [6]. Other, rarer forms of the disease include oculoglandular

[1] Category A select pathogens are those organisms/biological agents that pose the highest risk to national security and public health because they can be easily disseminated or transmitted from person to person, result in high mortality rates and have the potential for major public health impact, might cause public panic and social disruption, and require special action for public health preparedness (www.cdc.gov).

(infection of the eye), oropharyngeal (infection of the upper gastro-intestinal tract), and typhoidal (systemic infection without an ulcer).

The bacterium currently known as *F. tularensis* was first isolated from a ground squirrel in Tulare County, California, USA in 1911 [6] and since then *F. tularensis* has been isolated from more than 250 host species [7]. The severity of disease depends not only on route of exposure, but also on bacterial type [8]. *F. tularensis* is found throughout the Northern hemisphere and there are four recognized subspecies. The most virulent subspecies is *F. tularensis* subsp. *tularensis*, also known as "type A", and is found throughout North America. "Type B" tularemia is caused by *F. tularensis* subsp. *holarctica*, causes less severe disease, and is found throughout the Northern hemisphere. *F. tularensis* subsp. *mediasiatica* also generally causes mild disease, and is found in central Asia. The final subspecies, *F. tularensis* subsp. *novicida*, is considered avirulent in humans, and its classification is disputed. Chapter one of this thesis reviews the history of tularemia in wildlife and humans in the state of Alaska and describes the genetic characterization of recent Alaskan *F. tularensis* isolates.

Tularemia is an infectious disease of concern in Alaska and the Northern Hemisphere, and diagnosing this bacterial disease and others is important to both animal and human health. Numerous methods exist for surveillance of bacterial disease. However, none of them are truly unbiased or broad-based and able to detect a wide variety of bacterial species. Current methods for detecting past or current exposure to bacteria (including pathogens) include polymerase chain reaction (PCR), culture, and serology.

Bacteriologic culture is the gold standard for diagnosing most bacterial infections [9, 10]. Culturing confirms that live organisms are present, and determines biochemical and

phenotypic characteristics of the organism of interest. Despite being the gold standard, culturing is subject to limitations, namely that the bacteria in the sample must be alive and culturable to get a true positive result. Additional limitations include biosafety concerns, and that some bacteria are fastidious and require special growth media [2, 11].

Polymerase chain reaction (PCR) is a highly sensitive diagnostic tool that can determine the presence of bacterial DNA. The 16S ribosomal RNA gene is common to all bacteria. It encodes the 16S ribosomal RNA subunit, a structure essential for bacterial cell function. The gene contains regions that are highly conserved, allowing for the design of universal primers [12, 13]. It also has variable and hypervariable regions that allow for differentiation between bacterial taxa. This combination has made PCR, sequencing, and analysis of the 16S rRNA gene a very useful tool in bacterial phylogenetic analysis and is used extensively by microbiologists [14, 15]. Detection and sequencing of the 16S rRNA gene is beginning to be used by diagnosticians as well [16, 17, 18]. Chapter two of this thesis describes our development of both PCR and quantitative real-time PCR (qPCR) surveillance for bacterial DNA in animal tissue samples. We then used the technique to screen more than 1200 wildlife tissue samples for the presence of bacterial DNA and, in some cases to identify the bacteria present.

In chapter three, the broad-based PCR surveillance method developed above is used to investigate embryo mortality in greater white-fronted geese (*Anser albifrons*) on the North Slope of Alaska. Mechanisms of avian embryo mortality are poorly understood and recently microbial infection is being recognized as a cause [19, 20, 21]. Investigations into microbial sources of embryo mortality so far have focused on cavity-nesting tropical species and open-cup temperate nesting species, and none have focused on Arctic nesting

species. Bacterial egg pathogens are known to be horizontally and vertically transmitted [22, 23], and incubation has been shown to inhibit bacterial penetration of eggs in tropical environments [20], but there is evidence that these mechanisms act differently in temperate environments [24, 25]. We are unaware of any studies of avian embryo mortality in the Arctic.

During the summers of 2011 and 2012 approximately 10% of greater white-fronted goose eggs monitored in Northern Alaska contained at least one nonviable egg. This, coupled with the abundance of white-fronted geese in the area afforded us the opportunity to investigate embryo mortality in an Arctic nesting waterfowl species. The main objective of this study was to assess microbial infection as a source of avian embryo mortality in white-fronted geese on the Arctic Coastal Plain of Alaska. More specifically, we aimed to identify bacteria in the contents of nonviable eggs, compare bacteria present within the cloacae of nesting females, nest materials, and on eggshells to the contents of nonviable eggs, and to perform an infection study in a laboratory setting to asses pathogenicity and establish causality.

In addition to infectious disease, toxicologic diseases are a concern to animals and humans alike, representing an area of interest that is shared between human, veterinary and environmental health. Mercury is a nonessential element and a heavy metal of concern around the world, particularly in human populations that subsist on seafood and marine mammals [26, 27]. Mercury (Hg) is released into the atmosphere via natural and anthropogenic activities. Following entry to the atmosphere, microbial activity in ocean and lake sediments can transform organic Hg into the more toxic monomethylmercury ($MeHg^+$) [28, 29]. $MeHg^+$ is readily absorbed by lower trophic level biota and biomagnifies

up the food chain [30]. $MeHg^+$ reaches particularly high levels in some fish species and in piscivorous marine mammals [26, 27].

Clinical signs of Hg toxicity depend on dose and chronicity. Signs of acute toxicity (which is relatively rare and normally results from occupational exposure) include proprioceptive deficits, abnormal postures, blindness, anorexia, coma, and death [31]. More commonly seen, and of concern in wildlife, is chronic $MeHg^+$ exposure. There is evidence to show that chronic exposure to $MeHg^+$ can result in poor reproductive success, and impact behavior, cognition, and health [32, 33, 34]. Monitoring $MeHg^+$ exposure in wildlife is therefore important. Blood is the route of exposure for target organs (the central nervous system), and is therefore used in monitoring recent Hg exposure.

Collection of whole blood from wildlife in field situations can be problematic, particularly in remote locations with limited or no preservation or processing capabilities. Chapter four of this thesis describes the validation of a filter paper based sampling regime for the quantification of Hg in whole blood in two marine mammal species. This technique can be easily used in the field by scientists, hunters, fishermen, veterinarians or other trained personnel and will facilitate clinical and research monitoring efforts.

Taken together, these chapters highlight the importance of monitoring both infectious and noninfectious disease agents, and they describe new methods for surveillance. In addition, the development and implementation of two methods for assessing disease in wildlife will expand the toolbox of wildlife disease professionals. Chapter one describes a zoonotic disease of importance (tularemia) throughout Alaska and the Northern hemisphere. Chapter two describes development and implementation of a broad-based PCR surveillance and sequencing method for identifying bacterial DNA in

animal blood and tissue samples. Chapter three utilizes this broad-based PCR technique (and others) in an investigation of avian embryo mortality in the Arctic. Finally, chapter four describes and validates a novel method for monitoring mercury exposure in marine mammal whole blood.

In conclusion, simple and accurate methods to identify infectious and toxic agents in disease assessment may lead to a more comprehensive monitoring of wildlife health, which in turn might lead to interventions designed to improve wildlife health. According to the One Health paradigm, an improvement in wildlife health may also aid in improving human and environmental health.

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CHAPTER 1:

Tularemia in Alaska: 1938-2010[1]

Abstract

Tularemia is a serious, potentially life threatening zoonotic disease. The causative agent, *Francisella tularensis,* is ubiquitous in the Northern hemisphere, including Alaska, where it was first isolated from a rabbit tick (*Haemophysalis leporis-palustris*) in 1938. Since then, *F. tularensis* has been isolated from wildlife and humans throughout the state. Serologic surveys have found measurable antibodies with prevalence ranging from <1% to 50% and 4% to 18% for selected populations of wildlife species and humans, respectively. We reviewed and summarized known literature on tularemia surveillance in Alaska and summarized the epidemiological information on human cases reported to public health officials. Additionally, available *F. tularensis* isolates from Alaska were analyzed using canonical SNPs and a multi-locus variable-number tandem repeats (VNTR) analysis (MLVA) system. The results show that both *F. t. tularensis* and *F. t. holarctica* are present in Alaska and that subtype A.I, the most virulent type, is responsible for most recently reported human clinical cases in the state.

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1.1 Introduction

Tularemia is a serious and potentially life threatening zoonotic disease caused by the Gram-negative bacterium *Francisella tularensis*. Due to its high virulence and zoonotic potential, *F. tularensis* is listed as a category A select bioterrorism agent. *F. tularensis* has been weaponized in the past by the United States, Japan, the former USSR, and potentially other countries [1]. The organism was first isolated from a ground squirrel in 1911 in Tulare County, CA. It was named *Bacterium tularense*, was later reclassified as *Pasteurella tularense*, and finally, in 1966, was named *Francisella tularensis* after Edward Francis. Descriptions of a plague-like disease now considered to be tularemia predate this first isolation, going as far back as 1818 in Japan [2]. The first laboratory-confirmed human case was reported in 1914 [3]. Since then *F. tularensis* has been isolated from more than 250 host species [4].

F. tularensis is ubiquitous in the Northern hemisphere and currently there are four recognized subspecies. *F. tularensis* subsp. *tularensis* (type A) is the most virulent of subspecies and is found throughout North America. *F. tularensis* subsp. *holarctica* (type B) is less virulent and is found throughout the Northern hemisphere. The distinction between type A and B tularemia was first made in the middle of the 20th century [5]. Type A is divided into types A.I and A.II, and A.I is still further divided into types A.Ia and A.Ib. In a review of isolates collected in the US over 40 years, the highest human mortality rate was associated with type A.Ib (12/49 or 24%), followed by type B (8/108 or 7%), type A.Ia (2/55 or 4%), and finally, type A.II (0/53 or 0%)[6]. The third subspecies, *F. tularensis* subsp. *mediasiatica* is virulent and has been isolated in central Asia. Finally, many consider *F. tularensis* subsp. *novicida* to be a fourth subspecies of *F. tularensis* based on genetics and

biochemical requirements [7], though this classification is still disputed [8,9]. *F. tularensis* subsp *novicida* is generally avirulent in humans and is distributed globally [2,10].

The disease caused by *F. tularensis* depends on the route of entry. Ulceroglandular tularemia, the most common form of disease, results from exposure through the skin (either preexisting wound or arthropod bite). This form results in an ulcer at the site of infection followed by lymphadenopathy. Pneumonic tularemia, the most serious form of disease, results from inhalation of aerosolized bacteria. Other forms of the disease include oculoglandular (exposure via the eye), oropharyngeal (ingestion), and typhoidal tularemia (systemic infection without a primary ulcer).

Here we review the history of tularemia in both wildlife and humans in the state of Alaska. We also report on the genetic characterization of recent Alaskan *F. tularensis* human and animal isolates using canonical SNPs (canSNPs) and multi-locus variable tandem repeat (VNTR) analysis (MLVA).

1.2 Tularemia in Wildlife in Alaska

In Alaska, *F. tularensis* was first isolated from a rabbit tick (*Haemophysalis leporis-palustris*) removed from a varying hare (*Lepus americanus*) near Fairbanks in 1938 [11]. The isolated strain was virulent in both guinea pigs and rabbits, resulting in enlarged spleens and areas of focal necrosis in both the spleens and livers. The high virulence in both species suggests that the isolate may have been type A. Later, an additional two virulent and likely type A isolates were obtained when suspensions of ground ticks removed from two healthy hares were inoculated into guinea pigs [12,13]. Isolates collected from subsequent animals indicated the presence of a less virulent type, likely type B. The first of these was an isolate obtained from ticks collected from willow ptarmigan

(*Lagopus lagopus*) in the Fairbanks area in 1959 [14]. Voles sampled during the summer of 1963 on the Alaska Peninsula revealed a large number with splenomegaly and resulted in the isolation of another less virulent isolate [15]. During the summer of 1971 in the Fairbanks area, 10 of 24 hares had enlarged spleens from which *F. tularensis* was isolated [16]. This isolate was compared to the vole isolate from 1963 [15] and shown to be significantly more virulent in challenge studies, further supporting the coexistence of type A and B strains in Alaska [16] (Table 1.1).

Though few isolates have been obtained, serological surveys for tularemia conducted between 1964 and 2000 have indicated the presence of *F. tularensis* among a wide variety of wildlife species and across a wide geographic area in Alaska. Seropositive animals (titer ≥1:20) in these surveys included various rodents and hares, birds and large predators (Table 1.2). Of those titers reported, the range was 1:20 - 1:320 [14, 17, 18, 19, 20]. These serology results are consistent with the wide number of species in which *F. tularensis* has been found [4], but revealed few clues as to the important reservoir(s) for *F. tularensis* in Alaska. Of note, however, were two studies by Zarnke et al. [19,20], which found that positive tularemia serology peaks in predators followed peaks in snowshoe hare populations, suggesting the possibility of a hare reservoir. In addition, *F. tularensis* DNA was found in 30% of >2500 mosquitoes in Alaska, suggesting the possibility of an arthropod reservoir as well [21].

1.3 History of Human Tularemia in Alaska

The first possible case of human tularemia in Alaska was reported in 1938 in a 62-year-old man from Wiseman, north of the Arctic Circle. The patient exhibited symptoms of ulceroglandular tularemia and was hospitalized for 2 months, though there was no

laboratory confirmation of tularemia [13]. In 1946, a 31-year-old male from Northway (interior Alaska) with a history of skinning muskrats became the first laboratory-confirmed case by serology (titer 1:1280). His symptoms were headache, orbital pain, general aches and fever followed by development of swollen lymph nodes. The patient also reported that an ulcerated lesion had been present on his left middle finger for about one week. However, no isolate was cultured [22]. The first culture positive human infection occurred in 1974 in a 42-year-old laboratory worker with pneumonia whose pleural fluid yielded an isolate of *F. tularensis* [23].

Following the diagnosis of these initial cases of tularemia, surveillance projects were conducted throughout the state. The first of these occurred between 1954 and 1957 and involved 816 skin tests of inhabitants of Alaskan villages, of which 64 (8%) were positive, with 50 – 59 year olds having the highest incidence by age group [24]. The highest incidence was found in central Alaska, between Minto and Kaltag and as far north as Hughes, corresponding with the rich trapping areas in central Alaska. Following this initial surveillance, two additional surveys of Alaska Natives were completed. First, in the 1960s, serological surveys of 793 Aleut, Indian and Eskimo men showed an overall detection rate of 18% (139 of 793), with titers ranging from 1:20 to 1:640. A second survey involved skin tests on a subset of 115 (15%) of these Alaska Natives. Fifty-one (44%) of the 115 had positive skin tests, 43 (84%) of which also had detectable titers in the first survey, indicating a high correlation between skin test and titer results. Following these results, questionnaires were completed to determine if clinical disease resembling tularemia had been present. No difference in either total illness or tularemia-like illness was found

between seropositive and seronegative groups, suggesting that the tularemia present in Alaska Natives may be of a less virulent type [25].

A final survey of Alaska Natives was completed in 1974. In this study, there were 4% (29 of 810) and 7% (28 of 402) positive titer rates (≥1:80, range 1:80-1:1280) in southwestern and east central Alaska, respectively. In addition, two seroconversions in children were documented (both >4-fold increase in titer), with one child reporting a rash at around the time of the rise in titer and the other exhibiting no signs of disease. Similar to the previous surveys, no cases of tularemia-like illness were described in the villages studied, again suggesting that the tularemia present in these villagers was due to a less virulent type, that the route of infection favors asymptomatic disease or that Alaska Natives have developed resistance [23].

In 1993, two human cases related to housecats occurred in Fairbanks. One patient was a 44-year-old man who had been bitten on the thumb by his cat three and a half weeks prior to admission. Prior to the man's illness, his cat had been seen by a veterinarian and treated with antibiotics for an unknown febrile illness. The second patient was a 42-year-old veterinarian who presented with similar symptoms. The veterinarian had treated several cats with tularemia during the two-month period prior to his illness. Both human cases resolved with appropriate antibiotics [26].

Following the above housecat-associated cases, a serological survey of veterinarians was done in the Fairbanks area; two of 14 veterinarians (14%) had positive titers (≥1:80) for tularemia. Questionnaires sent to Fairbanks physicians and veterinarians indicated that 54% (15/28) and 92% (11/12), respectively, were aware that tularemia was prevalent in local wildlife. In addition, nine veterinarians had treated local domestic cats or dogs for

suspected tularemia, indicating that household pets can pose a significant source for human infection [26].

1.4 Epidemiology of Reported Human Cases in Alaska 1946-2010

Between 1946 and 2010, a total of 38 cases of tularemia were known to public health authorities in Alaska, with 9 cases in the Fairbanks-Steese area between 1946 and 1953 [27] and an additional 29 cases from throughout the entire state between 1972 and 2009. Of the 38 reported cases, 23 were laboratory confirmed, with detailed laboratory data available for 19 of those 23. Of these 19, 10 had four-fold changes in paired titers, 7 had positive cultures for *F. tularensis*, 1 had a positive lymph node stain and 1 had a single high titer along with clinical and epidemiological evidence. Seventy-three percent (22 of 30) of the patients were male with a median age of 39 years (range of 15-59 years). Seventy-one percent (27 of 38) were white and 16% (6 of 38) were of unknown race. Most (69%, 20 of 29) had illness onsets between June and August. Geographically, 68% (26 of 38) were exposed in central eastern Alaska, 21% (8 of 38) in the greater Anchorage area, 5% (2 of 38) in northwestern Alaska, 3% (1 of 38) in Southeastern Alaska and 3% (1 of 38) were exposed out-of-state. Ulceroglandular tularemia was most common (70%, 19 of 27), followed by typhoidal (11%, 3 of 27) and pneumonic (7%, 2 of 27) tularemia. None of the cases were fatal. Of those case-patients with detailed exposure histories, 79% (19 of 24) had direct contact with animals and 84% (16 of 19) of those had contact with a known wildlife reservoir (Figure 1.1). The remaining 16% (3 of 19) had had contact with domestic animals (one cat bite and two dogs known to have killed hares).

1.5 Molecular Subtyping of Recent *F. tularensis* Isolates

We subtyped DNAs from eight recent (2003-2009) *F. tularensis* isolates (6 hare and 2 human) obtained by the public health laboratory of Alaska from interior Alaska and an additional four Alaskan DNAs (3 human and 1 rodent) available in Northern Arizona University's *F. tularensis* DNA collection to determine if the presumed coexistence of types A and B in Alaska could be confirmed. We first subtyped the isolate DNAs using a set of canSNPs described by Vogler et al. [28] to identify the major *F. tularensis* subclades found in Alaska. We then subtyped the isolate DNAs using the MLVA system described by Vogler et. al. [29] in order to identify additional variation among the isolates.

The canSNP analysis identified 10 isolates as type A.I (6 hares, 1 rodent, 3 human), one as type A.II (human), and one as type B (human) (Figure 1.2). The canSNP assays further placed the type A.I isolates into subclade A.I.Br.001/002, the type A.II isolate into subclade A.II.Br.006/007 and the type B isolate into subclade B.Br.OR96-0246. This built upon a previous global analysis of *F. tularensis,* which had identified a single subclade A.I.Br.001/002 isolate (also included in this study) in Alaska [28]. This genetic analysis confirmed the previous virulence studies that had suggested the coexistence of types A and B in Alaska. Indeed, this analysis revealed an even greater level of genetic diversity than previously suspected, in that members of three major genetic groups were found to be present. The fact that most of these isolates were type A.I is likely related to the greater virulence of this genetic group [6] and thus the greater likelihood of severe disease and resultant opportunities for obtaining isolates through the public health system. However it is also possible that different strains are distributed differently throughout the environment, or that the reservoirs are distributed differently. It is probable that types B

and A.II are present in much higher proportions in the wild than is indicated by this analysis. By relying on clinical isolates for genetic analysis we are limited to strains that are more likely to cause disease. Intensive sampling efforts would be needed to obtain more isolates from wildlife or people in the state.

The MLVA analysis revealed additional genetic diversity among the Alaskan isolates. Specifically, a neighbor-joining analysis based on MLVA data for the Alaskan isolates and an additional 34 A.I.Br.001/002 isolates revealed that the Alaskan subclade A.I.Br.001/002 isolates did not form a monophyletic group. Rather, they were scattered amongst subclade A.I.Br.001/002 isolates from diverse North American geographic locations (Figure 1.2), indicating a relatively high level of genetic diversity within this subclade in Alaska. This relatively high level of genetic diversity suggests either multiple introductions of *F. tularensis* to Alaska, a long history of *F. tularensis* in Alaska with ample time for diversification and possible transfers to other geographic locations, or a combination of the two. However, it is important to note that such high levels of genetic diversity within a single geographic location are not unique to Alaska, having been observed elsewhere in North America [28]. Additional whole genome sequencing, SNP discovery and SNP screening as well as increased sampling will likely be needed to determine the origins and spread of *F. tularensis* in North America as a whole and Alaska specifically.

Interestingly, though there was no obvious geographic separation among the different Alaskan subclade A.I.Br.001/002 MLVA genotypes as they were all collected from Interior Alaska, the single Alaskan type A.II isolate was geographically separated from the other Alaskan isolates. The type A.II isolate (human) was isolated from the Matanuska Susitna Valley whereas most of the other isolates were from interior Alaska, where most

tularemia cases occur. These two regions are separated by the Alaska Range, which might serve as a geographic barrier separating type A.II *F. tularensis* from other *F. tularensis* genetic types in Alaska. However, this hypothesis would need to be confirmed by genotyping more isolates from both geographic regions.

1.6 Conclusions

We have reviewed a history of *F. tularensis* in Alaska, beginning with its first isolation in a group of hare ticks in 1938 and progressing to its molecular characterization in 2011. Only limited studies have taken place within the state, there is still much to be learned about the ecology and epidemiology of tularemia, particularly in northern climates where it is endemic. We still do not know the reservoir in Alaska, though it is suspected to be hares or muskrats. We also do not know the prevalence of tularemia in most of the wildlife in the state. Overall the presented work suggests the need for renewed serological surveillance in both wildlife and humans to assess possible changes in *Francisella* prevalence in a rapidly changing Arctic. The current distribution of tularemia in Alaska is not well understood. While most cases are reported from Interior Alaska, the true distribution of cases in wildlife and humans is not known. In addition more molecular work is warranted to better understand the strains circulating in Alaska and assess potential for human infection associated with different host species. Transstadial transmission of tularemia should be addressed similar to work done in Sweden [30]. These steps will further increase our understanding of tularemia in Alaska and can guide public health surveillance and intervention.

1.7 Competing Interests

The authors declare that they have no competing interests.

1.8 Author's Contributions

CH drafted the manuscript, AV and DM performed molecular analysis, PK edited the manuscript. KH conceived of the review and performed the literature search. All authors have read and approved the final manuscript.

1.9 Acknowledgments

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Table 1.1: Isolation of *Francisella tularensis* in Alaskan wildlife from 1938-1974

Year	Host	Location	# Positive	# Collected	Reference
1938	Rabbit Tick	Fairbanks	3 lots	3 lots	11
1953	Rabbit Tick	Minto, Livengood, Fairbanks	3 lots	14 lots	12
1960	Tick (from ptarmigan)	Livengood	1 lot	Unknown	24
1963	Red-backed vole	Alaska Peninsula	1	217	15
1971	Varying hare	Fairbanks	1	24	16

Table 1.2: Prevalence of *Francisella tularensis* antibodies (titer ≥1:20) in Alaskan wildlife from 1964 - 2000.

Year	Host	Location	#Positive	#Tested	Reference
1964	Dairy cattle	Tanana Valley	2	173	14
	Barrow ground squirrel	Tanana hills, Paxson	1	34	
	Red squirrel	Interior, Paxson	9	111	
	Red-backed vole	Interior, Paxson	2	120	
	Tundra vole	Interior, Paxson	11	229	
	Porcupine	Interior	1	2	
	Varying hare	Interior, Paxson	3	60	
	Cliff swallow	Interior	1	3	
	Bank swallow	Interior	1	38	
	Common redpoll	Interior, Paxson	1	15	
	Varied thrush	Interior	1	4	
	Northern water thrush	Tanana hills	1	3	
	American tree sparrow	Tanana hills	1	10	
	Willow ptarmigan	Tanana hills	1	2	
1967-68	Varying hare	Eagle	1	29	18
	Ground squirrel	Denali highway	2	72	
	Red-backed vole	Delta creek	1	376	
	Collared lemming	Nome	1	25	
	Wolf	Tok	1	15	
	Black bear	Circle hot springs	2	4	
	Marten	Eagle	9	26	
	Ermine	Katella	1	31	
	Lynx	Tok	1	4	
	Gray jay	Manley hot springs	2	19	
	Northern raven	Circle hot springs, Fairbanks	2	13	
	Northern shrike	Glenn highway	1	1	
1975-82	Wolf	Southcentral Alaska	16	67	19
1984-2000	Wolf	Southcentral Alaska	1	6	20
	Wolf	Central Interior	8	32	
	Wolf	Southern Interior	28	135	
	Wolf	Eastern Interior	2	30	
	Wolf	Western Interior	3	30	
	Wolf	Northern Interior	7	48	
	Wolf	Western arctic	5	75	
	Wolf	Eastern arctic	2	45	
1988-91	Grizzly bear	Kodiak island	3	77	17
	Grizzly bear	Alaska Peninsula	12	86	
	Grizzly bear	Interior Alaska	13	40	
	Black bear	Interior Alaska	13	40	
	Grizzly bear	Seward Peninsula	4	40	
	Grizzly bear	Noatak river drainage	12	87	
	Grizzly bear	Arctic northwest	34	96	
	Grizzly bear	Arctic northeast and central	15	54	

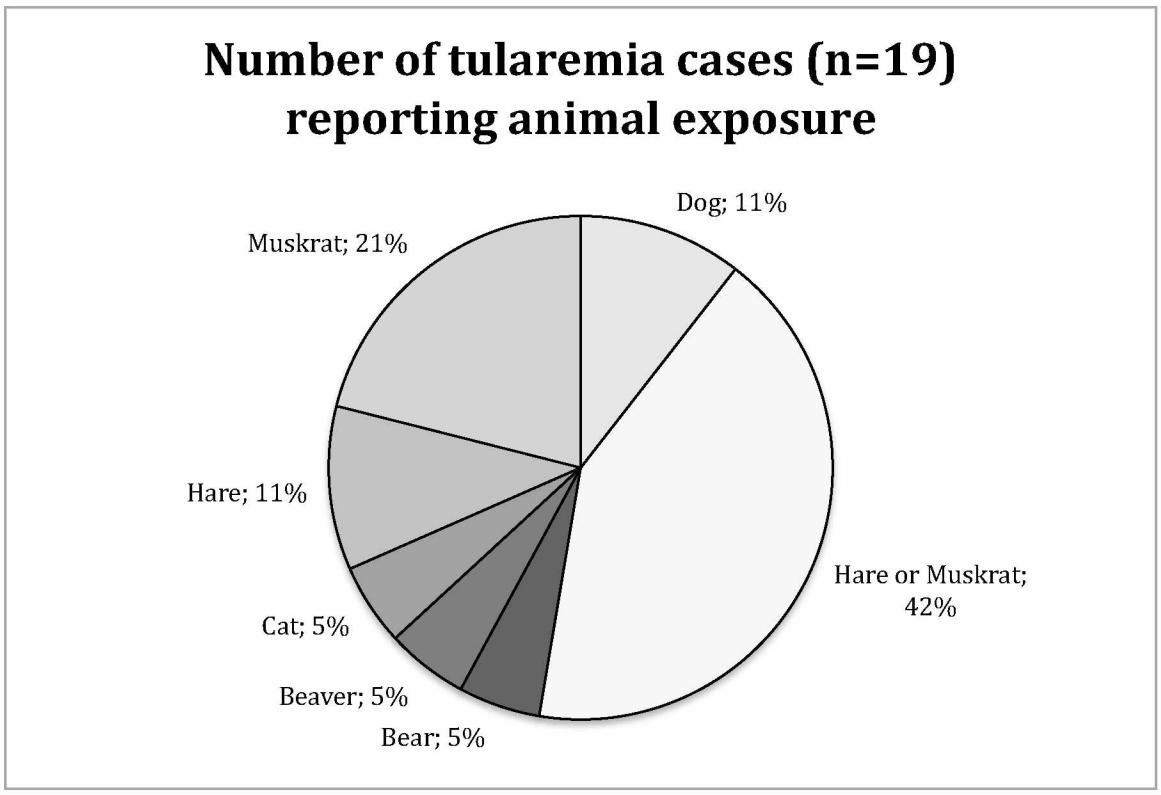

Figure 1.1: Number of human tularemia cases in Alaska reporting animal exposure.

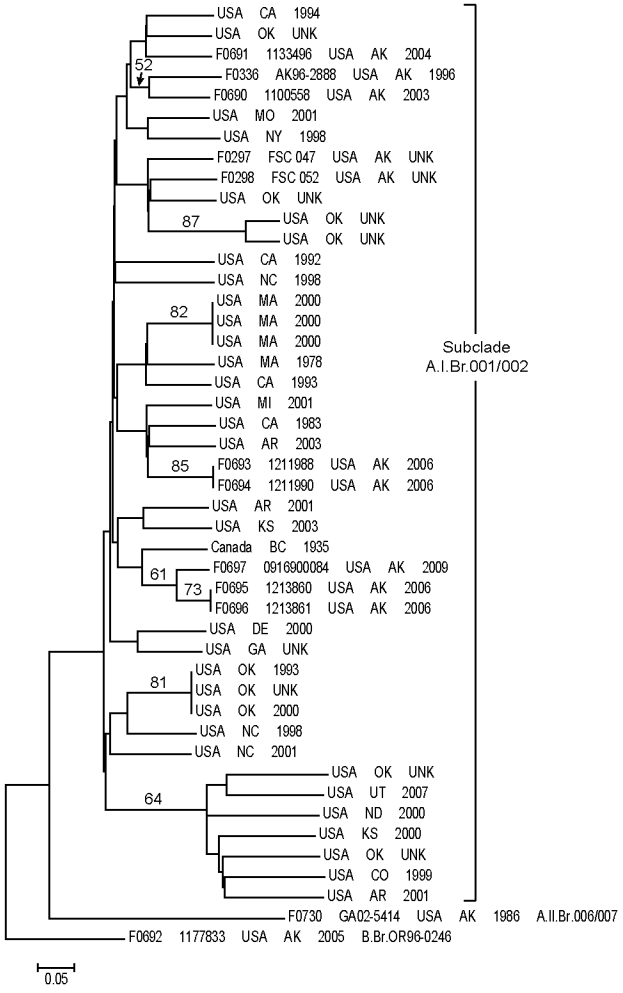

Figure 1.2: Neighbor-joining dendrogram of Alaskan and 34 additional subclade A.I.Br.001/002 *F. tularensis* isolates based upon MLVA data. The dendrogram was generated using neighbor-joining analysis of mean character differences using PAUP 4.0b10 (D. Swofford, Sinauer Associates, Inc., Sunderland, MA). Bootstrap values ≥50, also generated using PAUP 4.0b10, are indicated and were based upon 1,000 simulations.

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CHAPTER 2:

Development and implementation of a broad-based polymerase chain reaction surveillance method for bacterial DNA in Alaskan wildlife tissues[1]

Abstract

Current methods for bacterial exposure surveillance in animal blood and tissues include agent-specific antibody assays (usually for serum), culture, and agent-specific polymerase chain reaction (PCR) based assays. For most of these methods a specific disease agent is suspected or targeted before diagnostic or survey tests are conducted. In the current study, the development and implementation of PCR-based protocols to broadly survey (non-targeted) bacteria using detection and sequencing of the bacterial 16S ribosomal RNA (rRNA) gene in opportunistically collected tissue samples of wildlife in Alaska are described. Quantitative real-time PCR was used to survey large sets of wildlife tissues (n=844). End-point PCR (n=454) was used on smaller sets of tissue samples. Of 1298 samples, 108 had detectable PCR product; upon sequencing, 65 revealed interpretable sequences without the need for cloning, 47 of which are known or suspected pathogens. More sequences from potentially pathogenic bacterial species were detected in necropsy specimens, but likely contaminants originating from the gastrointestinal tract were detected as well. These results show that 16S rRNA gene-based PCR methods are potentially valuable tools for performing large-scale non-targeted surveillance for bacterial pathogens with recognition of limitations for strain or highly specific identification.

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2.1 Introduction

Current methods for establishing the presence of bacteria in tissue samples include (but are not limited to) targeted detection of specific taxa using polymerase chain reaction (PCR), and cultivation, which is considered the gold standard for disease diagnosis in many pathogenic species of bacteria.[22,37] Additionally, specific reactive antibody detection (serology) can be used to detect current or past exposure to certain types of bacterial organisms. Limitations of these techniques include antibody cross reactivity and persistence of titers at detectable levels, which can make it difficult to diagnose an active (rather than historic) infection (serology);[19,30,41] and prolonged time to obtain results (culture).[19,41] It should be noted that most serum antibody detection and targeted PCR tests are agent-specific in that primers and antigens need to be selected and generated prior to performing diagnostics tests. There are multiple reasons for performing diagnostic tests on wildlife samples. The presence of or exposure to a specific organism may to be suspected, alternatively, there may be a compelling public health or food safety reason to monitor for that agent.

Serology allows for detection of active infection or past exposure, and is an invaluable tool for assessing exposure of populations to pathogens. However, determining if the organism(s) in question is present at sampling based on serology can be difficult.[19,41] Typically early in an initial active infection (1-2 weeks) no specific antibody is detectable;[13,30] the immunoglobulin M titer then begins to rise and can remain elevated during active or recent infection. The immunoglobulin G titer rises during convalescence and can remain high for many years or a lifetime.[30] Antibody cross reactivity (e.g. *Brucella* and *Yersinia*) adds additional uncertainty to interpreting serologic results.[9,11,25] Finally, antibody detection often relies on species-specific

reagents, and not all assays are validated for the host species and associated bacteria for which they are used.[17]

The gold standard for diagnosing most active bacterial infections is culture and isolation of the causative agent.[22,37] Culture confirms that live organisms are present in the tissue of interest. Culture methods are also used to compare biochemical and phenotypic characteristics of type strains to the isolate to be identified.[7] However, due to diversity beyond that recognized by phenotypic identification schemes, an unambiguous identification cannot always be made, resulting in uncertain identification of the isolated organism or the need for follow-up tests to arrive at a final diagnosis. Some bacteria are fastidious, and diagnostic laboratories may not be equipped to culture them or may not attempt isolation.[36] Organisms must also be viable in order to be successfully cultured. In most clinical specimens viability is typically not a concern, however, in poorly preserved tissue specimens, freezer archives, or when shipping long distances, this requirement may be problematic to achieve. Biosafety and biosecurity also must be considered when growing certain organisms (e.g. *Bacillus anthracis, Francisella tularensis, Yersinia pestis*), limiting diagnostic culture capabilities in some facilities.[14,29]

Polymerase chain reaction (PCR) is a commonly used and highly sensitive diagnostic tool that can rapidly detect or identify bacterial DNA through sequencing of amplicons. Multiplexing has led to tests that can diagnose multiple pathogens simultaneously.[2] Further, PCR can be used to amplify any part of a bacterial genome, and commonly amplified segments include regions coding for plasmids, virulence factors, and membrane proteins.[10,38] While these genes can be used to quickly identify certain pathogenic bacteria, none of these are common to most bacterial species. The use of a broad-based PCR surveillance method for bacterial DNA using a universal target is worthy of exploration, development, and ultimately strategic implementation.

The 16S rRNA gene is common to all bacteria and encodes the 16S ribosomal RNA subunit. This gene is present in eukaryotic mitochondria as well, but its sequences are clearly divergent from bacterial 16S rRNA genes so that primers that amplify most bacterial 16S rRNA gene sequences will not amplify those from eukaryotic mitochondria.[27] The bacterial 16S rRNA gene is approximately 1,542 base pairs in length and is critical to bacterial cell function. The gene contains regions that are highly conserved allowing for selection of broad-based primers. It also contains regions that are variable or hypervariable, providing opportunities for sensitive differentiation between bacterial taxa.[5,20] Characterization of conserved and variable regions has made sequencing and analysis of the 16S rRNA gene a useful tool in bacterial phylogenetic analyses.[20]

The 16S rRNA gene is used extensively in microbiological applications; most notably in the fields of microbial ecology and in determining phylogenic relationships of bacteria and archea.[34,39] Additionally, PCR and sequencing of the 16S rRNA gene has been used in applications to identify human bacterial pathogens and is integral to the study of the human microbiome.[12,18] Two studies examined the usefulness of broad ranged 16S rRNA targeted primers in the diagnosis of septic arthritis,[3,31] and one examined an application to the field of human endodontics.[15]

A similar 16S rRNA gene PCR technique has been used to identify an animal pathogen,[16] and some veterinary diagnostic laboratories are using 16S rRNA gene amplification to identify bacteria in tissue samples, and data from some published surveillance efforts exist.[8] It is important that we monitor wildlife populations for bacterial disease agents, as the majority of emerging human disease events originate in wildlife.[21] Additionally, wildlife tissue sample collection is often done opportunistically and the amount of tissue collected can be limited.

Therefore it is important to develop surveillance tools that are broad-based and can detect more than one type of bacterial disease agent.

In the current study, both endpoint and quantitative real-time PCR (qPCR) techniques and their application to disease surveillance in diverse taxa of wildlife are described. These techniques use universal primers that amplify the 16S rRNA gene from bacteria present in certain tissues that should not normally contain bacteria in healthy (uninfected) individuals (e.g. spleen, liver, lymph node, kidney, reproductive tract) as an indication of infection or contamination. The 16S rRNA gene PCR products from both end-point and qPCR were partially sequenced without cloning to determine what bacterial taxon was present in positive samples.

2.2 Materials and Methods

2.2.1 Sample Collection

A total of 1298 blood and tissue samples were utilized for this survey, see Table 2.1 for a summary of samples. Tissue samples from apparently healthy foxes were collected opportunistically from trappers and hunters throughout Alaska. Harbor seal samples (blood) were collected under the National Marine Fisheries Service (NMFS) scientific research permit #358-1787 and sampling was performed with the approval of the Alaska Department of Fish and Game Animal Care and Use Committee (ACUC) approval (#07-16 modified/renewed). Dolphin samples (blood) were collected under NMFS scientific research permit #522-1785 and sampling was performed with approval of the Mote Marine Laboratory Institutional ACUC (#08-09-RW1, and 09-09-RW1). Ice seal samples (tissues) were collected under permit #932-1905-00/MA-009526 issued by the NMFS and the U.S. Fish and Wildlife Service (USFWS). California sea

lion and harbor seal samples (tissue) were collected under the Alaska SeaLife Center's National Oceanic and Atmospheric Administration/National Marine Fisheries Service (NOAA/NMFS) stranding agreement. Additionally, necropsy specimens from the Alaska Department of Fish and Game (ADF&G), University of Alaska Fairbanks (UAF) Animal Resources Center, and local veterinary clinics were included when opportunistically available. Samples collected included spleen, liver, lymph nodes, kidney, or any lesion where infection was suspected (i.e. granuloma or abscess). Samples were collected aseptically in the field or at UAF or ADF&G facilities by wildlife veterinarians or UAF veterinary staff (from the Animal Resource Center). The foxes from trappers in remote areas were submitted frozen and skinned, and abdominal and thoracic cavities remained unopened until sampling. Information on individual foxes was not provided, so time from skinning to freezing, and from freezing to sampling is unknown.

Since samples were collected opportunistically and culture was not performed as an integral part of this study, culture results were only present for some tissues. Cultures were performed on ADF&G samples by Colorado State University Veterinary Diagnostic Laboratories (Ft. Collins, CO, USA). There was one culture-positive tissue from a local veterinary clinic; this culture was performed at Fairbanks Memorial Hospital (Fairbanks, AK, USA).

2.2.2 DNA Extraction

The DNA was extracted from tissue samples using a commerical kit[a] following manufacturer's instructions. Samples were processed and analyzed as they were received. For large batches of samples (n>50), DNA was extracted using a 96-well kit[a]. One formalin-fixed tissue from a local veterinary clinic was pre-treated by rinsing with phosphate buffered saline as per the manufacturer of the kit's instructions. The remaining samples required no pretreatment, as per

manufacturer's instructions. To reduce the effects of potential PCR inhibitors,[30,39] blood DNA was diluted 1:10 in nuclease free water; liver, spleen, and other tissues DNA were diluted 1:20 in nuclease free water prior to PCR.

2.2.3 Primers

Primers were selected flanking the 16S rRNA gene (see Table 2.2 for primer sequences) and were purchased from a commercial supplier.[b] Forward primer F2C and reverse primer R2C were used for the initial amplification reaction[1] for both PCR and qPCR. This primer set amplifies an approximately 1,500 base pair portion of the 16S rRNA gene.

2.2.4 End-point PCR Reactions

End-point PCR reactions were performed on small batches of samples (n<48) as they arrived. Each PCR reaction contained one commercial ready-to-go PCR bead,[c] 18 µl of nuclease-free water, 10 pmol of each primer F2C and R2C, and 5 µl of diluted DNA template for a total reaction volume of 25 µl. Reactions were performed using a thermal cycler[d]. Thermocycler settings for end-point PCR reactions were as follows: 3 minutes of denaturation at 94°C followed by 40 cycles of 30 seconds of denaturation at 94°C, 1 minute annealing at 65°C, and 1 minute 40 seconds of extension at 72°C. A final 10-minute 72°C extension step was followed by hold at 4°C until the reaction tubes were collected.

One positive and one negative control were amplified with each end-point PCR reaction. *Escherichia coli* (*E. coli*) DNA diluted in liver, blood, or spleen DNA (depending on sample type being analyzed) was used as a positive control. Blood came from an apparently healthy reindeer housed at the UAF Large Animal Research Station. Liver and spleen DNA was extracted from

the liver and spleen of a disease-free rat housed at the UAF Animal Resources Center. Negative controls were selected to match the sample type being tested (blood, liver, or spleen DNA, as above). Some tissue samples were not commonly received (e.g. lymph node, kidney, uterus/testes); liver DNA was used as a negative control in these cases.

Five µl of PCR reaction product was run on 1% agarose gel using standard methods in Tris-acetate-ethylenediaminetetraacetic acid (TAE) buffer. Each gel was stained for 20 - 30 minutes in TAE buffer containing ethidium bromide, imaged with ultraviolet light and the image captured with a digital camera. Positive samples showed a sharp band at 1,500 base pairs.

To estimate concordance between end point PCR and qPCR, 58 DNA samples that were positive (and yielded a sequence) with end-point PCR were run in qPCR as described below.

2.2.5 qPCR Reactions

Quantitative real-time PCR conditions were optimized separately from end-point PCR. For large batches of samples ($n \geq 48$), and for concordance testing, qPCR reactions were performed. Each qPCR reaction contained 10.5 µl of master mix,[e] 7.5 pmol of each primer, 3 µl of nuclease free water, and 5 µl of diluted DNA template for a total reaction volume of 20 µl. The qPCR reactions were run either in a 384-well clear optical reaction plate[f] on a commercial system[g] or in 96-well format on another commercial system.[h]

The thermal cycler settings for the qPCR assay were as follows: 10 minutes of denaturation at 95°C, 40 cycles of 15 seconds denaturation at 95°C, 30 seconds of annealing at 60°C, and 2 minutes 20 seconds of extension at 72°C. This was followed by a melting curve; 95°C for 15 seconds, 60°C for 15 seconds, and ramping to 95°C for 15 seconds. Fluorescence

data was collected during each annealing phase and during the determination of the melting curve.

The qPCR data were analyzed using commercial software.[i] The qPCR reactions were considered positive if the cycle threshold (C_t) value was $\geq$2 lower than the lowest of the negative controls for each run. Dissociation (melting) curves were also used to interpret positive qPCR reactions: samples with a single sharp peak (in addition to meeting C_t criteria) were considered reliably positive.[30] Peaks with a shoulder and double peaks were considered negative.

Positive controls consisted of DNA from either *E. coli* or *Francisella tularensis* (*F. tularensis*) subsp. *novicida* DNA diluted in either nuclease free water, liver DNA, blood DNA, or spleen DNA. Negative controls were the same as for end-point PCR. In addition to these negative controls, no-template-controls (nuclease free water) were included with every qPCR run, see detection limit section below.

2.2.6 qPCR Detection Limit and Dilution Factor Determination

E. coli (DH10B)[j] was grown overnight in Luria broth (LB) at 37° C, and *F. tularensis* subsp. *novicida* (ATCC 15482) was grown in tryptic soy broth (TSB). The following day, 100 µl of each overnight culture was subcultured (in their respective medium) and grown to 1 (+/- 0.05) optical density at a wavelength of 650 nm on a spectrophotometer.[k] From these broth cultures, eight serial ten-fold dilutions were prepared. Ten µl of each dilution was plated on LB agar or TSB agar, respectively, and the plates were incubated overnight. The following day colony numbers were counted and colony forming units/ml were calculated. Additionally, DNA was extracted from 1 ml of each serial dilution using a commercial kit[a] according to the manufacturer's instructions. Each extraction was eluted into 200 µl of final volume. The DNA

concentration and purity of each standard was determined once using absorbance at 260 nm and the ratio of absorbance at 260 and 280 nm on one of two spectrophotometers,[l,m] The DNA extracted from these serial dilutions was used as standards in qPCR analysis and were used to calculate the detection limit in ng/reaction. The detection limit was defined as the smallest amount of bacterial DNA detectable in 95% of reactions.[4]

The presence of PCR inhibitors such as hemoglobin, glycogen, and fats[30,39] necessitates the dilution of sample DNA. To determine the optimal dilution factor, serial dilutions of bacterial DNA in nuclease free water (*E. coli* or *F. tularensis*) were added to different dilutions of negative DNA control (1:1, 1:10, 1:20, 1:50, or 1:100) obtained from different tissues and 20 replicate qPCR reactions were carried out to determine a 95% detection limit, as above.

2.2.7 Sequence Analysis

Primers and excess dNTPs were removed from the PCR products with a commercial kit[n] according to the manufacturer's instructions. Sanger sequencing of purified PCR products was performed by a commercial service.[o] Positive samples were first sequenced with one internal reverse primer (R1). If the sequence from that primer appeared reliable (high base quality scores) and if the initial sequence aligned with a potential pathogen in BLASTn, additional sequencing was performed using primer F1 to increase sequence coverage and quality. Only consensus sequences of overlapping regions of sequences were used for sequence identification of those sequenced with more than one primer. A complete list of all primers (PCR and sequencing) is shown in Table 2.2.

Sequence results were manually checked for quality by inspecting electropherograms, then trimmed using Ridom TraceEdit (http://www.ridom.de/traceedit/). Sequences with single

peaks in each position on chromatograms were considered interpretable and were exported as text files for further alignment and analysis. Those sequenced with more than one primer (R1 and F1) were aligned using clustalw (http://www.ebi.ac.uk/Tools/msa/clustalw2/) using slow alignment type and default settings. A consensus sequence was generated for samples sequenced with more than one primer. The consensus sequence was defined as the sequence that was in common between two primers. If a discrepancy at a base pair was noted, chromatograms were examined and the base with higher calling score was selected to use in the consensus sequence. Sequences were entered into the National Center for Biotechnology Information (NCBI) Basic Local Alignment Search Tool (BLASTn) algorithm (http://blast.ncbi.nlm.nih.gov/) for identification. The 16S rDNA database was searched in BLASTn and the program was optimized for highly similar sequences. The BLASTn database was searched between June 2011 and June 2013. A minimum cutoff identity score was not used, the highest identity sequence score was taken as the best match for the sequence obtained.

2.3 Results

To assess the sensitivity of amplification of the complete 16S rRNA gene by qPCR, a detection limit was determined by amplifying this gene from two different bacterial species. Extracted DNA from *E. coli* and *F. tularensis* subsp *novicida* was diluted in extraction products from different tissue matrices used in this study. The detection limit (Fig. 2.1) for *E. coli* DNA diluted in nuclease free water was 0.64 ng/reaction, which corresponds to 1.29×10^5 genomes (calculated). This method detected 0.4 pg/reaction of *E. coli* DNA (8 genomes) in 75% of the qPCR runs. Considering the dilution of samples, this (0.4 pg/reaction) corresponds to about 2.6×10^6 genomes per ml in the original culture prior to DNA extraction. The 95% detection limit for

E. coli DNA spiked in a 1:10 dilution of blood DNA was 4 ng/reaction, diluted in 1:20 liver DNA was 40 ng/reaction, and diluted in 1:20 spleen DNA was 4 ng/reaction. These detection limits correspond to 1.6×10^8, 3.2×10^9, and 3.2×10^8 bacterial genomes per gram of original tissue when mass of tissue used for the extraction and dilutions are considered.

The detection limit for *F. tularensis* subsp *novicida* DNA diluted in water was 0.1 ng/reaction (4.6×10^4 genomes). This method detected 0.01 ng/reaction of *F. tularensis* DNA (5×10^4 genomes) in 50% of the qPCR runs. Diluted in 1:10 blood DNA the detection limit was 1 ng/reaction (4.6×10^5 genomes), in 1:20 liver DNA was 10 ng/reaction (4.6×10^6 genomes), and in 1:20 spleen DNA was 10 ng/reaction (4.6×10^6 genomes). These 95% detection limits correspond to 9.3×10^7, 1.9×10^8, and 1.9×10^8 genomes per gram of original tissue. Similar detection limits were obtained for both *F. tularensis* and *E. coli* using two separate qPCR instruments (the same number of runs was not completed with each qPCR machine). Based on this detection limit data, it was determined that the optimal dilution factor for blood was 1:10, and for liver and spleen was 1:20.

A total of 225 wells of nuclease free water (no template control) were run throughout the course of this surveillance effort. In total, 62% (n=141) of the wells did not cross the baseline fluorescence threshold after 40 cycles. The average C_t value of the remainder of the no template controls (n=84) was 36.74 and none had a discernable melting curve. In addition, negative control samples (liver, spleen, and blood DNA) did not have sharp dissociation curves, therefore no negative control sample met our inclusion criteria for a positive sample.

Out of all PCR reactions (end-point and qPCR) (n=1298), 108 (8%) had detectable PCR product (Table 2.3). Of those 108 positives, 65 (60%) yielded interpretable sequences. Of the 65 interpretable sequences, 48 sequences most closely matched sequences from obligate or

opportunistic pathogens. The 17 sequences not considered pathogens were sequences that most closely matched soil bacteria (5 sequences had 97-99% identity matches with an uncultured *Burkholderia* identified from soil, GenBank accession JQ400905.1), or were best matched with uncultured clone sequences.

Among end-point PCR reactions (n=454), 83 (18%) had detectable PCR product (visible band at 1,500 bp), 52 of those (63%) had interpretable sequences. Of the 52 sequences, 47 were highly similar to known or opportunistic pathogens. Among qPCR reactions (n=844), 25 (3%) had detectable PCR product (C_t value more than 2 below lowest negative control, single peak dissociation curve), 13 of those (52%) had interpretable sequences. Of those 13 sequences, 1 (8%) closely matched known or opportunistic pathogens. Table 2.4 lists closest matches for sequences obtained in this study. There were more poor quality or very short sequences in qPCR (n=12 or 50% of total sequences), relatively fewer with endpoint PCR (n=43 or 40% of total sequences). All sequences except for one (a 307 bp sequence with 90% identity to *Clostridium nexile*) had identity scores $\geq$95%. Fifty of 65 sequences (77%) obtained using R1 and/or F1 had identity scores $\geq$97%.

The health status of the host was determined by reviewing necropsy reports and veterinary medical records. Animals captured live, or from which samples were collected for herd health assessments were presumed to be generally healthy. Health status was listed as either infectious disease suspected (n=84) or infectious disease not suspected (n=1214). The qualification 'infectious disease' included all disease with infectious etiology, not just bacterial etiologies. Sequences were obtained from 30% of tissues where infectious disease was suspected, and from 3% of tissues where infectious disease was not suspected (Table 2.5). Sequences matched opportunistic or obligate pathogen sequences in 93% (27/29) of animals suspected of

having infectious disease. In non-infectious disease suspects, this number was lower (58% or 21/36).

Of those 58 samples that yielded good quality sequences using end-point PCR, 32 (55%) were positive using qPCR. Of those 32, 23 had interpretable sequences (72%). Of those, 17 (74%) had matching sequences (genus and species), 3 more (13%) had matching sequences at the genus level, and 3 (13%) of the sequences did not match those obtained from endpoint PCR.

Concordance was evaluated in all cases where possible, but most tissue samples did not have culture results available, as samples were collected opportunistically. In several cases culture or targeted PCR did confirm the results obtained by the PCR-sequencing method in the tissues tested. In 4 cases *F. tularensis* sequences were obtained from samples (snowshoe hare liver and spleen) that had previously been confirmed positive with targeted (*Francisella*-specific) PCR.[14] Additionally, a guinea pig lung sample that was culture-confirmed positive as *Bordetella bronchiseptica* yielded *B. bronchiseptica* sequences with both end-point and qPCR techniques. Three samples yielding *Streptococcus uberis* sequences were cultured and sequenced with *Streptococcus* specific primers and confirmed to be *S. uberis*. Finally, the same organism was confirmed in different tissues (from the same animal) in several instances. For example, in two moose, *E. coli* was cultured from lung and kidney, and the technique described in this study obtained *E. coli* sequences from the spleen and a lymph node, an additional moose lung was culture positive for *P. multocida,* and a *P. multocida* sequence was obtained using the universal primers described here. These results are consistent with what would be expected in a systemic infection.

There were occurrences of PCR results in the same or different tissues not matching results from culture. In a rabbit lung sample, *E. coli* was cultured but a sequence most closely

matching *Neisseria meningitidis* was obtained using end-point PCR. In a mountain goat, lung and lymph node cultured *Trueperella pyogenes*, but *Streptococcus uberis* sequences were obtained using end-point PCR. In one mountain goat sample, mixed culture results (*Enterobacter* sp., *Enterococcus, E. coli, Acinetobacter* sp., *Actinomyces*, and *Streptococcus*) were obtained from both lung and lymph node, and the end-point PCR based technique described here yielded a sequence closely matching *C. perfringens* in the lung and *S. uberis* in the lymph node. In this case the *C. perfringens* is most likely a post mortem contaminant. Finally, in two moose *E. coli* was cultured from the lung and a *C. sordellii* sequence was obtained from one lymph node and a *Streptococcus macedonicus* sequence was obtained from the other's lymph node using end-point PCR.

2.4 Discussion

This investigation has shown that broad-based 16S rRNA gene amplification and sequencing can be a valuable technique when used under the right circumstances for use in surveillance of bacteria that are potentially pathogenic in populations of wildlife. The PCR methods described here are particularly useful when infectious disease is suspected (i.e. necropsy or clinical specimens) in a normally sterile body tissue as a part of standard surveillance. In many cases, interpretable DNA sequences are obtained without the need for cloning or culture. In addition, both techniques are rapid and cost effective when applied to sampling efforts that involve large numbers of samples.

Previous studies have used similar 16S rRNA gene-based techniques to diagnose and identify pathogens in limited applications. None have used similar techniques for broad surveillance as applied here. As early as 1994, a PCR based assay was used to detect pathogens

in cerebrospinal fluid.[12] Two qPCR methods to amplify the 16S rRNA gene to determine the etiology of septic arthritis have been reported.[3,31] Additionally, fluorescent dyes and melting curve analysis have been used to differentiate between 17 species of Gram-positive and Gram-negative bacteria[23]. Both Taq Man- and SYBR green-based qPCR techniques have been used to investigate bacterial concentrations in endodontic samples[15]. Finally, universal 16S rRNA gene primers have been used to amplify a *Streptococcus phocae* sequence from the uterine exudate in a spotted seal with pyometra[16].

Various C_t cutoff levels can be utilized. Two previously mentioned qPCR studies[3,31] used C_t cutoff values of >1 value below negative control, which is less stringent than the threshold in this study. The lower cutoff value used in the current study ($\geq$2 C_t values), while raising the detection limit, will decrease the number of false positives, and still identify clinically relevant bacterial loads. The detection limits in tissue determined in this study are higher than in agent specific qPCR assays.[26,36] The higher detection limit may be due to the long PCR product (1,500 bp, leading to less efficient PCR). In addition, the broad range of these primers can lead to amplification of small amounts of contamination resulting in a lower C_t value of negative controls. This lower C_t value of negative control samples will increase the amount of template DNA needed to cross the threshold at 2 C_t below the negative sample. Despite this adjustment, the detection limits obtained in this study do correspond with what is quantified in clinical infection.[7,24,29]

Additionally, qPCR can be very sensitive, and at high cycle numbers most samples will cross the threshold, either due to amplification of fragments of eukaryotic DNA, or amplification of *E. coli* DNA in *Taq* polymerase.[33] For this reason PCR using universal primers will not be as specific as agent-specific PCR for some bacterial taxa. Additionally, although the selected

primers amplify a broad range of bacterial taxa, they are not strictly universal. Based on the Ribosomal Database Project probe match function (http://rdp.cme.msu.edu/probematch), the F2C, R2C primer pair will amplify 16S rRNA genes from 2,052 (of 3,662) complete bacterial 16S rRNA sequences in that database (accessed 12/4/2013).

The initial primer set used in this study amplified nearly the entire 16S rRNA gene. If a sample was positive, internal primers were used to amplify a smaller segment (approximately 500 bp) of the gene. This shorter sequence (which includes 3 variable regions) was used in our identification. While a minimum of 500 bp of sequence is recommended for microbial identification (1,300 to 1,500 is ideal),[20] the technique presented here allows for screening and preliminary identification of bacterial DNA in tissues. If 500 bp sequences are obtained and aligned with less than 1% ambiguity to strains available in public nucleotide databases, bacterial species can be identified with confidence.[20] If further identification is desired (short or ambiguous sequences), further sequencing using universal or specific primers can be performed.[16]

Additionally, not all variable regions within the 16S rRNA gene are able to distinguish between different species of bacteria, and there is no single variable region that is able to distinguish among all bacteria.[5] Regions V1, V2, and V3 (amplified by F1/R1 primer set) are able to distinguish between most pathogenic bacterial species, but were not able to distinguish between some *Escherichia* species, *Shigella* species, *Klebsiella pneumonia*, and *Enterobacter aerogenes*.[5] If sequencing these regions reveals a member of one of these genera, additional sequencing may be required to attempt to identify to the species level. However, it should be emphasized that for the host species and geographic regions targeted for this surveillance the need for all positive samples to be identified to the bacterial species or strain level is not required.

One would likely follow up when a linkage with a disease outcome is of management concern (public health, conservation) or scientific interest, an agent that may be emerging and worthy of further investigation, or a potential agent of biosecurity interest (foreign animal disease, bioterrorism). This is the value of the non-targeted, unbiased nature of this effort.

Previously published literature has not attained 100% concordance between culture and qPCR, or between species-specific targeted PCR and 16S rRNA gene qPCR, meaning that the same species is not always identified using multiple methods.[3] Coinfection, or infection plus a contaminating bacterial organism are likely responsible for some of these differences. As expected, this study did not find 100% concordance between sequence data and the limited culture results that were available.

Additionally, this study examined concordance between end point PCR and qPCR on a limited number of available samples and did not arrive at 100% concordance. It is not surprising that differing PCR protocols, large amplicon size, and additional freeze-thawing (especially in conjunction with large amplicon size)[31] may contribute to the somewhat limited concordance noted between end point and qPCR here. The end-point PCR and qPCR protocols developed during this study were optimized individually. Reagents used in each system are different and different optimal thermal profiles are to be expected thus limiting an interassay comparison of suitable rigor. These results do show, however, that both systems (end-point PCR and qPCR) can be adapted for use in a broad-based 16S rRNA gene PCR protocol.

It should be noted that only 3% of qPCR reactions (n=844) were determined to be positive based on C_t value and dissociation curve while 18% of end point PCR reactions (n=454) were considered positive based on presence of a clear band at 1,500 base pairs. This is expected, as the samples run in the qPCR protocol came from large collections of trapped or live sampled

(presumably healthy) animals. Small batches of samples provided by the Alaska Department of Fish and Game were from necropsy specimens of sick or injured wildlife, thus it is not surprising that more of these samples contained bacterial DNA from that sample pool. This batching was based on logistics of sample receipt and timely testing and underscores the fact that the percent of positive samples in the two assays cannot be compared to assess performance of the two assays.

Also, it should be noted that not all samples from animals suspected of having an infectious disease yielded a positive PCR result. This is because animals suspected of having infectious disease of any etiology (viral, bacterial, or fungal) were included in this group. It is likely that some of these animals had viral, fungal, or noninfectious disease, which cannot be detected by this assay. Alternatively, animals might have been infected with a bacterial species whose 16S rRNA gene could not be amplified by the primer set used in this study. Finally, some of these results are simply false negatives. In most cases, data from culture or agent specific PCR assays were not available so we could not quantify a false negative rate. The lack of culture data is due to the opportunistic nature of sample acquisition that was performed in collaboration with a variety of individual and institutions.

The major advantages to this broad-based technique are that it is not agent-specific (non-targeted), it is cost effective, fast, and has potential for very high throughput and relatively fast turnaround. Using the protocols developed for this study, the total cost per sample (including extraction, PCR, and sequencing) is less than $7.00 per sample. Including DNA extraction, positive/negative status can be obtained in less than 12 hours with either of these PCR techniques. If sequencing is available on-site, sequencing data takes several more hours. Sequencing was done off site in this study and results were available within 48 hours. The data presented here

show that the universal primers used will amplify many obligate and opportunistic pathogen 16S rRNA genes but will also amplify the 16S rRNA genes from gastrointestinal or other contaminants. Additionally, with on site sequencing results are available less than 24 hours after sample acquisition. As such it is suited to large survey efforts utilizing a batch approach.

Similar to interpreting other diagnostic tests, context must be considered when interpreting sequences obtained with either of these PCR techniques. In some cases, a sequence represents an obligate pathogen (e.g. *F. tularensis*) and should be interpreted as an abnormal result, but many bacteria are opportunists and only cause disease when the host is compromised, such as when the immune system is impaired. In support of this, it is noted that more sequences from opportunistic pathogens (i.e. *Clostridium* species) were identified in animals that were not considered infectious disease suspects (Table 2.4). Similar to the gold standard of culture, sequencing results must be interpreted in the context of clinical and environmental factors.

One further limitation is the inability to easily resolve coinfection or contamination with multiple bacteria. Cloning PCR product into a plasmid vector and performing a restriction fragment length polymorphism assay or next generation sequencing would aid in resolving this issue, but that was not within the aims and scope of this investigation.

In conclusion, the two PCR-based techniques discussed above show potential use in non-targeted surveillance and monitoring despite the limitations discussed above. The equipment required to perform end-point PCR is inexpensive and commonplace. qPCR equipment may be cost prohibitive. This approach (using one or both methods) would be most beneficial as an initial screening tool. Depending on sample size, end-point or qPCR could be used. Following initial PCR and sequencing; cloning, culture, or targeted PCR could be adopted, depending on

the research or management question related to the surveillance efforts to obtain more specific results.

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2.6 Sources and Manufacturers

a. DNeasy Blood and Tissue Kit, Qiagen, Valencia, CA

b. Eurofins, Huntsville, AL

c. illustra PuRe Taq ready to go PCR bead, GE Healthcare, Pittsburgh, PA

d. MJ Mini, Bio Rad, Hercules, CA

e. SYBR Green Real-Time PCR Master Mix, Applied Biosystems, Foster City, CA

f. MicroAmp Optical 384-Well Reaction Plate with Barcode, Applied Biosystems, Foster City, CA

g. 7900HT Fast Real-Time PCR System, Applied Biosystems, Foster City, CA

h. 7500 Fast Real-Time PCR System, Applied Biosystems, Foster City, CA

i. SDS 2.2.2, Applied Biosystems, Foster City, CA

j. Invitrogen, Grand Island, NY, USA

k. Genesys 10 uv vis, Thermo Fisher Scientific, West Palm Beach, FL

l. NanoDrop 1000, Thermo Fisher Scientific, West Palm Beach, FL

m. DU Series 700, Beckman Coulter, Fullerton, CA

n. SV Wizard PCR Clean-up Kit, Promega, Fitchburg, WI

o. Elim Biopharmaceuticals, Hayward, CA

2.7 Declaration of Conflicting Interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Table 2.1. Summary of all samples included in this broad-based polymerase chain reaction surveillance project. Sequence obtained refers to the number of 16S ribosomal RNA sequences obtained from that species and tissue type.

Species	Tissue	n	Sequences obtained	Infectious disease suspected[a]
American beaver (*Castor canadensis*)	Lymph node	2		
American black bear (*Ursus americanus*)	Lymph node	2		
	Spleen	2	1	
American mink (*Mustela vison*)	Unknown	5		5
Arctic fox (*Vulpes lagopus*)	Lymph node	1		
	Spleen	1		
Arctic ground squirrel (*Spermophilus parryii*)	Liver	1		1
Bottlenosed dolphin (*Tursiops truncatus*)	Blood	11		
California sea lion (*Zalophus californianus*)	Blood	1		1
	Lymph node	2		2
Canadian lynx (*Lynx canadensis*)	Spleen	5		3
Caribou (*Rangifer tarandus*)	Amniotic fluid	1		
	Blood	2		
	Joint fluid	1		
	Kidney	3	1	1
	Liver	1		
	Lung	3	1	2
	Lymph node	45	4	5
	Mammary gland	1		
	Peritoneal fluid	1		

Table 2.1 continued

	Spleen	53	3	6
	Testes/uterus	11	1	
	Tonsil	1		1
Common raven (*Corvax corvax*)	Spleen	1		1
Coyote (*Canis latrans*)	Lymph node	2		
	Spleen	4		
Dall sheep (*Ovis dalli*)	Lymph node	5	3	1
	Spleen	11	1	1
Domestic cat (*Felis catus*)	Cystic fluid	1		
	Uterine material	1	1	1
Domestic dog (*Canis lupus familiaris*)	Blood	1		1
	Uterine material	1		
Domestic guinea pig (*Cavia porcellus*)	Lung	2	2	2
Ermine (*Mustella erminea*)	Spleen	1		
European rabbit (*Oryctolagus cuniculus*)	Lung	1		1
Great-horned owl (*Bubo virginianus*)	Spleen	2	1	1
Grizzly bear (*Ursus arctos*)	Lymph node	3	2	
Harbor seal (*Phoca vitulina*)	Blood	144	5	
	Lung	2		
Little brown bat (*Myotis lucifugus*)	Lung	1		
	Spleen	2		2
Moose (*Alces alces*)	Blood	27		
	Joint fluid	1		
	Lymph node	16	3	7
	Spleen	11	3	4

Table 2.1 continued

	Unknown	1		1
Mountain goat (*Oreamnos americanus*)	Lymph node	4	1	3
	Spleen	2	1	2
Muskox (*Ovibos moschatus*)	Lymph node	3	1	3
	Spleen	4	2	2
Red fox (*Vulpes vulpes*)	Liver	355	4	1
	Lymph node	10		1
	Muscle	1		
	Spleen	363	4	2
Red squirrel (*Tamiasciurus hudsonicus*)	Spleen	2		
Reindeer (*Rangifer tarandus*)	Liver abscess	1	1	1
Ringed seal (*Phoca hispida*)	Liver	5		
	Lung	1		
	Lymph node	25		
	Spleen	8		
	Testes	1		
	Thymus	3		
	Urine	1		
Sitka black-tailed deer (*Odocoileus hemionus*)	Lymph node	1		1
Snowshoe hare (*Lepus americanus*)	Liver	7	3	6
	Spleen	3	1	1
	Unknown	1	1	1
Spotted seal (*Phoca largha*)	Amniotic fluid	1		
	Liver	2		
	Lymph node	6		

Table 2.1 continued

	Spleen	2		
	Urine	1		
Swainson's thrush (*Catharus ustulatus*)	Liver	1		1
Wolf (*Canis lupus*)	Blood	22		
	Kidney	7		2
	Liver	3		1
	Lung	6	1	1
	Lymph node	15	1	
	Spleen	25	10	1
Wolverine (*Gulo gulo*)	Liver	1		
	Lymph node	1		
	Spleen	2		
Wood bison (*Bison bison*)	Lymph node	1		1
	Spleen	2	1	1
Woodchuck (*Marmota monax*)	Spleen	1		

[a]Infectious disease suspects were determined by reviewing necropsy reports and veterinary medical records. Infectious diseases of any suspected etiology were included. Numbers in this column are numbers of disease suspects.

Table 2.2: Primers used for amplification and sequencing of the 16S ribosomal RNA gene

Primer	Sequence (5' – 3')	Direction	Position[a]	Ref
F2C	AGAGTTTGATCCTGGCTCAG	Forward	8	1
R2C	AAGGAGGTGATCCANCCRCA	Reverse	1541	1
F1	GAGTTTGATCCTGGCTCAG	Forward	9	26
R1	GWATTACCGCGGCKGCGG	Reverse	500	12

a. Primer numbering relates to *Escherichia coli* 16S rRNA gene nucleotide position from the 5' end of the primer

Table 2.3: Overall polymerase chain reaction (PCR) and quantitative real-time PCR results. Positive PCR status was determined by the presence of a band at 1500 base pairs under ultraviolet light after staining with ethidium bromide. Positive qPCR status was determined by cycle threshold value ($\geq$ 2 less than negative control) and by the presence of a single peak dissociation curve. Sequence indicates how many interpretable sequences were obtained from positive samples, pathogen indicates if the sequence was associated with an obligate or opportunistic pathogen.

	PCR (n=454)	qPCR (n=844)	Total (n=1298)
Positive	83 (18%)	25 (3%)	108 (8%)
Sequence	52 (11%)	13 (2%)	65 (5%)
Pathogen	47 (10%)	1 (0.1%)	48 (4%)

Table 2.4: Closest identities of sequences obtained by species and tissue. Sequence length is also provided. Organisms were identified by searching sequences using the National Center for Biotechnology Informations (NCBI) Basic Local Alignment Search Tool (BLAST).

Organism	Species	Tissue[a]	Sequence Length	Disease suspect (Yes,No)
Bordetella bronchiseptica	Guinea pig	Lung	456	Yes
Clostridium	Wolf	Spleen	193	No
Clostridium bartletti	Red fox	Spleen	420	No
Clostridium butyricum	Muskox	Spleen	433	Yes
Clostridium haemolyticum	Red fox	Liver	444	No
Clostridium haemolyticum	Caribou	LN	415	No
Clostridium haemolyticum	Caribou	Kidney	438	No
Clostridium haemolyticum	Wolf	Spleen	315	No
Clostridium haemolyticum	Wolf	Spleen	399	No
Clostridium haemolyticum	Wolf	LN	407	No
Clostridium haemolyticum	Wolf	Spleen	224	No
Clostridium nexile	Caribou	Uterus	307	No
Clostridium perfringens	Red fox	Spleen	336	No
Clostridium perfringens	Mountain goat	Spleen	409	Yes
Clostridium perfringens	Great horned owl	Spleen	335	No
Clostridium perfringens	Wolf	Lung	248	No
Clostridium perfringens	Caribou	Spleen	436	Yes
Clostridium septicum	Muskox	LN	433	Yes
Clostridium sordellii	Black bear	Spleen	423	No
Clostridium sordellii	Moose	LN	420	Yes
Clostridium sordellii	Grizzly bear	LN	350	No
Clostridium sordellii	Dall sheep	LN	372	No
Clostridium sordellii	Wood bison	Spleen	318	Yes
Clostridium sordellii	Dall sheep	LN	426	No

Table 2.4 continued

Clostridium sordellii	Caribou	LN	400	No
Escherichia coli	Caribou	Spleen	464	Yes
Escherichia coli	Caribou	LN	460	Yes
Escherichia coli	Moose	Spleen	449	Yes
Escherichia coli	Moose	LN	443	Yes
Francisella tularensis	Snowshoe hare	Spleen	455	Yes
Francisella tularensis	Snowshoe hare	Liver	438	Yes
Francisella tularensis	Snowshoe hare	Liver	506	Yes
Francisella tularensis	Snowshoe hare	Unknown	516	Yes
Fusobacterium spp.	Wolf	Spleen	104	No
Fusobacterium necrophorum	Reindeer	Liver abscess	429	Yes
Fusobacterium necrophorum	Muskox	Spleen	423	Yes
Fusobacterium necrophorum	Guinea pig	Lung	212	Yes
Neisseria meningitidis	European rabbit	Lung	270	Yes
Pasteurella multocida	Moose	Spleen	463	Yes
Pasteurella multocida	Wolf	Spleen	372	No
Pasteurella multocida	Moose	Spleen	358	Yes
Pasteurella multocida	Caribou	Lung	48	Yes
Pasteurella multocida	Caribou	LN	440	Yes
Streptococcus spp.	Grizzly bear	LN	151	No
Streptococcus uberis or *ictaluri*	Dall sheep	Spleen	216	Yes
Streptococcus uberis or *ictaluri*	Dall sheep	LN	313	Yes
Streptococcus uberis or *ictaluri*	Mountain goat	LN	444	Yes
Streptococcus macedonicus	Moose	LN	460	Yes

[a]LN = lymph node.

Table 2.5: Sequences identified from tissues suspected of having infectious disease vs. tissues not suspected of having infectious disease.

Health Status	n	Interpretable sequences obtained	Suspect pathogen sequences obtained
Infectious disease suspect	84	29	27
Not infectious disease suspect	1214	36	21

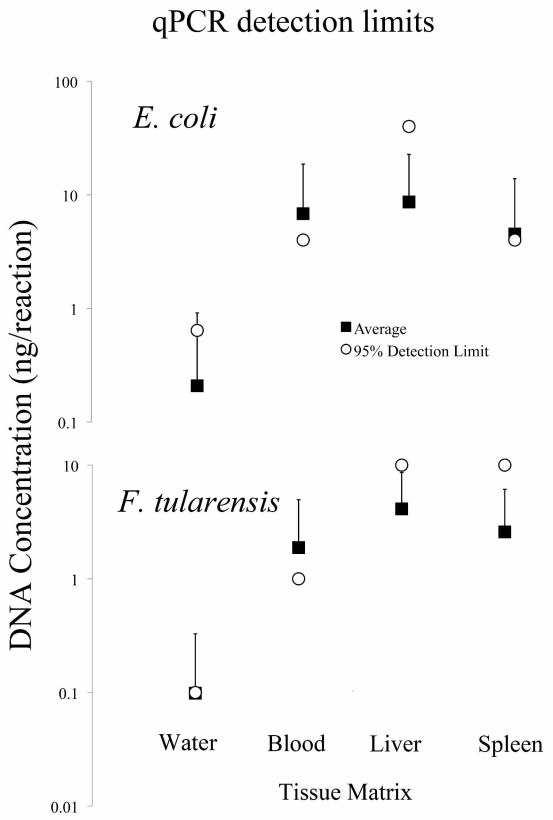

Figure 2.1. Detection limits for *Escherichia coli* and *F. tularensis* DNA in water and various tissue matrices. Solid squares indicate the average detection limit; open circles are the 95% detection limit (lowest concentration detected in 95% of quantitative real-time polymerase chain reactions) from 20 replicate runs. Error bars represent one standard deviation from the mean.

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Chapter 3

Microbial infection as a source of embryo mortality in wild geese on the Arctic Coastal Plain of Alaska[1]

Abstract

Whereas it is established that microbial infection is a source of embryo mortality in avian eggs, much remains unknown regarding processes acting on hatchability in wild birds. To address the role of bacterial infection as a cause of hatching failure in wild geese, we monitored embryo development in a breeding population of greater white-fronted geese (*Anser albifrons*) on the Arctic Coastal Plain of Alaska. During 2013, we observed mortality of developing embryos and collected 36 apparently addled eggs for analysis. We used standard culture methods and 16S rRNA gene sequencing to taxonomically identify bacteria within collected eggs. A potentially novel species of *Neisseria* was isolated from 23 eggs, *Macrococcus caseolyticus* was isolated from 6 eggs, *Streptococcus uberis* and *Rothia nasimurium* were each isolated from 4 eggs. Other bacterial taxa were isolated at lower frequencies. Sequences of the 16S rRNA gene from our *Neisseria* isolate most closely matched sequences from *N. animaloris* and *N. canis* (96-97% identity), but phylogenetic analysis suggests substantial genetic differentiation between egg isolates and known *Neisseria* species. Additionally, we detected DNA from our *Neisseria* isolate in 44 of 63 egg shell and nest swab samples, and in 4 of 28 cloacal swab samples. To assess the pathogenicity of bacteria identified in contents of addled eggs, we inoculated our isolates of *Neisseria*, *Macrococcus*, *Streptococcus*, and *Rothia* of varying concentrations into developing

[1] Hansen CM, Meixell BW, Van Hemert CR, Hare RF, Hueffer K. 2014. Microbial infection as a source of embryo mortality in wild geese on the Arctic Coastal Plain of Alaska. Prepared for submission to Applied Environmental Microbiology.

chicken eggs. Seven-day mortality rates varied from 60-100%, depending on species and dose of inoculum. Our results provide the first evidence of bacterially-induced embryo mortality in both wild geese and in the Arctic.

3.1 Introduction

Egg hatching failure causes a direct reduction in avian reproductive success and represents a cost to individual fitness. Despite the importance of egg hatchability to fecundity in birds, the mechanisms of embryo mortality remain poorly understood. Viability of avian eggs declines as the time to incubation onset increases (1) and prolonged exposure to ambient temperatures was traditionally considered the primary factor responsible for this pattern (1-3). However, recent research suggests that microbial infection may be an important proximal mechanism of embryo mortality (4-6). Investigations of microbial processes acting on bird eggs have been historically limited to cavity-nesting species in tropical climates (4, 5, 7), but more recently expanded to include temperate climates and open-cup nests (6, 8, 9).

Potentially pathogenic bacteria may be transmitted from the cloaca or reproductive organs of the nesting female, or may originate in the environment (e.g., nest bowl, 10) and enter the egg through the shell via pores. A variety of bacteria are present on eggshells shortly after laying, and subsequently grow to maximum abundance after three days (6). The presence of water on eggshells increases the abundance and diversity of bacteria present, and appears to play an important role in sustaining bacterial growth (11). In tropical environments, there is a positive relationship between trans-shell infection and humidity and temperature, and incubation inhibits bacterial growth and trans-shell penetration by reducing moisture on shells (5, 12). Furthermore, Godard et al. (6) reported considerably higher bacterial loads on eggs in open-cup nests as compared to those in cavity nests, likely as a result of increased exposure to water in the former. In contrast, recent research suggests that in temperate climates with cooler ambient temperatures, incubation may not hinder bacterial growth or penetration of eggshells (9, 13).

Less commonly, direct vertical transmission from the nesting female may result in infection or mortality of eggs. For example, there is no direct relationship between eggshell contamination with *Salmonella enterica* Enteritidis (SE) and contamination of egg contents, indicating that contamination may occur in the reproductive tract (14). Additionally, it is possible to isolate SE from the reproductive tracts of hens without fecal colonization (14). Contamination of the albumin is thought to occur as the egg passes through the oviduct. *Campylobacter* (reviewed in 15) and *Mycoplasma* (16) are less well studied, but are also thought to be vertically transmitted in avian eggs.

Hatching failure resulting from nonviable eggs has been commonly reported in temperate- and arctic-breeding waterfowl (17), however, the effects of microbial processes on waterfowl egg hatchability in northern regions have not been investigated. During the summers of 2011 and 2012, we identified at least one nonviable egg in approximately 10% of greater white-fronted goose (*Anser albifrons*) nests monitored on the Arctic Coastal Plain of Alaska (B. W. Meixell, unpublished data). While no previous nonviable egg rates exist for this nesting population, it is high compared to other populations of greater white-fronted geese (hereafter: white-fronted geese) in the Arctic (17). This, along with the abundance of nests in the area provided us the opportunity to investigate hatching failure in this population.

White-fronted geese breed in Alaska and Northern Canada and winter in the Southern and Western United States and Mexico (18). White-fronts have historically nested in low densities on the Arctic Coastal Plain of Alaska, but over the last 20 years have increased dramatically to become the most abundant nesting waterfowl species in the area (19). Furthermore, the Arctic Coastal Plain represents the northern extent of their breeding

range. White-front nests usually contain 3-6 eggs, with the female typically initiating incubation upon laying the penultimate egg (18). The egg mortality rate in this nesting population of white-fronted geese is high compared to that in other populations (17). While we do not know the cause of mortality in these eggs, based on the appearance of addled eggs, we hypothesize that some mortality is due to microbial infection.

The primary objective of this study was to assess microbial infection as a source of embryo mortality in white-fronted geese on the Arctic Coastal Plain of Alaska. Specifically, we sought to identify bacteria in contents of nonviable eggs, compare bacteria present within the cloaca of nesting females, in nest materials, and on eggshells to those found in the contents of nonviable eggs to assess potential sources, and inoculate embryonated chicken eggs with bacterial isolates from nonviable eggs to assess pathogenicity and establish causality.

3.2 Materials and Methods

3.2.1 Sample Collection. We monitored nests and collected samples between June 14 and July 14, 2013 near Point Lonely, Alaska on the Arctic Coastal Plain (70° 54' 45.49" N, 153° 14' 28.82" W). We located white-front nests on foot. Each nest was assigned a unique ID and its location was recorded with a handheld GPS unit. Each egg was candled to determine incubation stage (20) and individually labeled with a number corresponding to laying order based on egg staining (21); eggs were labeled on both ends with a permanent marker. We visited nests every 4 – 7 days, at which time each egg was candled to identify embryo mortality and estimate hatch date. Eggs that were noted to be addled (dead or not developing with a history of containing a viable embryo) were collected and transported on

foot to the field camp where contents were aspirated immediately. Eggs suspected of being infertile (no indication of development after a known period of incubation) were collected for comparison.

We collected egg contents by disinfecting the surface of the eggshell with 70% isopropyl alcohol, puncturing the air sac end and aspirating up to 2.0 mL of egg contents with a syringe and needle. Egg aspirate samples were transferred to sterile cryovials and aspirate and whole egg samples were kept at -20°C for up to 15 days until shipment to the University of Alaska Fairbanks (UAF), where they were stored at -50°C. A subset of eggs (n=7) collected in the 48 hours prior to leaving the field site were transported chilled on ice packs to UAF and cultured immediately upon arrival. Whole egg samples were thawed in the lab, opened and contents were examined visually to determine if eggs were addled or infertile.

We swabbed eggshells and nest contents to evaluate possible bacterial sources. We used BD liquid Amies elution swabs (Eswab, BD, Franklin Lakes, NJ, USA) following the manufactures instructions and swabbed approximately 1/3 of the surface of each eggshell and multiple locations within the nest. During egg-laying, prior to incubation onset, we selected a random sample of nests (n=12) from which we swabbed both egg shells and nest contents separately. During incubation, we used a single swab to sample nest contents and eggshells from nests identified as containing an addled or infertile egg; for comparison, we also selected and swabbed eggshells and nest contents from nearby nests that contained only viable eggs. We also obtained cloacal swab samples from a subset of nesting females during late incubation. We captured birds on nests using bow-net traps (22) and collected cloacal samples by inserting a swab approximately 10 mm into the cloaca. Swabs were kept

cool on ice packs and transported on foot to the field camp where they were frozen at -20°C within a few hours of collection. Swabs were shipped frozen to UAF, where they were stored at -50°C until thawed for analysis.

All procedures were approved by the U.S. Geological Survey Alaska Science Center Animal Care and Use Committee and were authorized by U.S. Fish and Wildlife Service and Bureau of Land Management under permit numbers MB789758 and BLM AK FF095718, respectively.

3.2.2 Bacterial Culture and Identification. Sequencing and culture results from 3 eggs collected during a pilot study in 2011 demonstrated that an approximately 350 base pair region of the 16S rRNA gene closely matched 3 bacterial genera (*Neisseria*, *Staphylococcus*, and *Macrococcus*), two of which were grown in culture (*Staphylococcus* and *Macrococcus*). However, some *Neisseria* species are fastidious and require specialized growth media (23); additionally, these eggs had been frozen for an extended time, possibly contributing to negative culture results. Using these preliminary sequence results, we tailored our culture protocols to target the putative *Neisseria* genus of bacteria while still allowing for *Staphylococcus* and *Macrococcus* to grow. Egg aspirates were plated on chocolate agar and blood agar (Remel, Lenexa, KS, USA) and incubated at 37°C for a minimum of 24 hours. Any plates that showed no growth after 24 hours were left in the incubator for up to 72 hours and checked for bacterial growth daily.

3.2.3 Microscopy. After isolation into pure culture, each colony type was Gram stained using standard methods and photographed under a light microscope. For transmission electron microscopy, bacterial cultures were grown in tryptic soy broth. The culture was transferred to a Formvar® coated copper 200 mesh grid (SPI Supplies West

Chester, PA) for one minute to allow bacteria to settle, and then culture was blotted off with filter paper. The bacteria that adhered to grids were stained with 1% phosphotungstic acid for one minute, which was removed by blotting with filter paper. Grids were washed with water and then air-dried. Images were taken on a JEOL JEM-1200EX TEM operated at 85 kV and AMT Image Capture Software (version 5.4.2.247) and an Orca (Hamamatsu) 12 bit 1024 by 1024 bit CCD camera.

3.2.4 DNA Extraction. In order to perform PCR, DNA was extracted from 25 µL of egg aspirate samples using a Qiagen DNeasy blood and tissue kit (Qiagen, Valencia, CA, USA) following manufacturer's instructions for animal tissue samples. DNA was extracted from 1 mL of overnight culture in tryptic soy broth following the manufacturer's instructions for Gram-negative bacteria. DNA was extracted from swab samples by placing the liquid medium in a microcentrifuge tube and centrifuging at 5,000 x *g* for 10 minutes. The supernatant discarded and the remaining pellet was resuspended in 180 µL buffer ATL (tissue lysis buffer) and DNA as extracted using the Qiagen kits following the manufacturer's instructions for tissue extraction. DNA was stored at -50°C until analysis.

3.2.5 PCR and Sequencing. To amplify the bacterial 16S ribosomal RNA gene we used PCR of egg direct aspirate and pure culture samples. To further genetically characterize our *Neisseria* isolate, we performed additional PCR and sequencing of the chaperonin 60 (cpn60) gene. All PCR reactions used illustra PuRe Taq ready-to-go PCR beads (GE Healthcare, Pittsburgh, PA, USA) and were performed in an MJ Mini personal thermal cycler (Bio Rad, Hercules, CA, USA). Positive and negative controls were run alongside samples. *E. coli* DNA was used for positive controls for 16S rRNA and cpn60 gene PCR reactions. *Neisseria* DNA isolated from one of the eggs was used as a positive control

for *Neisseria* detection PCR. Nuclease free water was used as a negative control for all reactions. The *E. coli* (positive control) rRNA gene was amplified in all runs of our 16S rRNA and cpn60 gene PCRs. The *E. coli* rRNA gene was not amplified in any run of our *Neisseria* detection PCR. All negative controls were negative. We performed PCR for the 16S rRNA gene using the "universal" primers F2C and R2C (44). Thermal cycling parameters were as follows: 94°C for 180s followed by 40 cycles of 94°C for 30s, 65°C for 60s, 72°C for 100s. A final 10 minute 72° extension phase was followed by a 4° indefinite hold. These primers amplify the bacterial ribosomal RNA gene from a wide variety of bacterial species (13,388 bacterial 16S rRNA sequences as of 11/21/2013 using the Ribosomal Database Project's Probematch; http://rdp.cme.msu.edu/probmatch). Bacteria were identified via sequencing the PCR product and identified using the National Center for Biotechnology Information's basic local alignment search tool (BLAST).

Chaperonin 60 gene PCR was performed using the primers H529 and H530 (24) to further genetically characterize the *Neisseria* isolate. This gene is present in prokaryotes and eukaryotes and has a finer phylogenetic resolution than the 16S rRNA gene (reviewed in 24). Thermal cycling parameters were as follows: 94°C for 180s followed by 40 cycles of 94°C for 30s, 50°C for 60s, 72°C for 60s. A final 10 minute 72° extension phase was followed by an indefinite 4° hold.

Additionally, we designed primers cpn2F (5'-AGCCGGTACCTGAAAAGTCA-3') and cpn2R (5'-ACAGGCAGCAAATCACGGATA-3') to amplify a 304 bp portion of the cpn60 gene in the novel *Neisseria* isolate detected in this study. This *Neisseria*-specific PCR was used on our swab samples in an attempt to identify the source of the bacteria. In addition to our broad-based 16S rRNA gene PCR to detect bacterial DNA, this *Neisseria*-specific PCR

protocol was used on all originally obtained egg aspirate samples to detect *Neisseria* DNA in any other samples. Thermal cycling protocols were as follows: 94°C for 3 minutes followed by 40 cycles of 94°C for 30s, 68°C for 60s, 72°C for 30s. A final 10 minute 72° extension step was followed by an indefinite 4° hold.

Sanger sequencing of PCR product was performed by Elim Biopharmaceuticals (Hayward, CA). The 16S rRNA gene PCR products were initially sequenced using primer R1 (5'-GWATTACCGCGGCKGCTG-3'), which obtains approximately the first 500 base pairs of the 16S rRNA gene (25). We used additional primers to obtain a full length 16S rRNA gene sequence for some of our *Neisseria* isolates: primers F1 (5'-GAGTTTGATCCTGGCTCAG-3') (26), F2D (5'-GATTAGATACCCTGGTAG-3') (27), and R2B (5'-CTTGTGCGGGCCCCCGTCAATTC-3') (28). Chaperonin 60 sequences were obtained using primers M13F and M14B (29). Sequences were manually inspected using Ridom Trace Edit (www. ridom.de/traceedit) and trimmed to remove poor quality base scores at the ends of each sequence. Sequences were considered uninterpretable if more than one peak was present at each nucleotide position on the chromatogram. To improve coverage and generate consensus sequences for samples sequenced with multiple primers, sequences were aligned using Clustal omega (www.ebi.ac.uk/Tools/msa/clustalo/). Phylogenetic trees were generated after aligning and trimming sequences using SeaView (www.molecularevolution.org/software/alignment/seaview). Because cpn60 sequence data are not available publicly for *Neisseria canis* (a close relative of our isolate), we obtained and sequenced *N. canis* type strain H6 (ATCC14687) from the American Type Culture Collection (Manassas, VA, USA) for comparison.

3.2.6 Embryonated Egg Infections. In an attempt to fulfill Koch's postulates we inoculated chicken embryos with the bacteria isolated most commonly from our eggs as described by Nix et al. (30). Two of our novel *Neisseria* isolates and one isolate each of *Macrococcus caseolyticus*, *Streptococcus uberis*, and *Rothia nasimurium* were grown to the late log phase (optical density at 600 nm, 0.95 to 1.05) and diluted in phosphate buffered saline (PBS) for injection. Inoculating doses were determined through serial dilution and colony-forming unit (CFU) counts, five 10-fold dilutions were prepared from each isolate. One-day-old fertilized White Leghorn chicken eggs obtained from Charles River Labs (Wilmington, MA) were incubated at 37°C with high humidity and mechanically tilted to a 45° angle every hour for seven days prior to infection and throughout the experiment. Five eggs were inoculated with each dilution of each strain (125 total eggs) and 9 control eggs were inoculated with PBS. Eggshells were punctured at the air sac end and 100 µL of inoculum was injected under the chorioallantoic membrane with a tuberculin syringe. After injection, the shells were sealed with a drop of Elmer's® School glue (Elmer's Products Inc. Columbus, OH).

The viability of the embryos was determined via candling and eggs were checked daily starting one day after infection for 7 days (i.e., when embryos were 14 days old). We scored embryos that lost the integrity of their capillary networks as dead (30). A sample of egg contents from each egg was plated on tryptic soy agar the day that they died, or at day 7 post-infection for survivors and controls. All plates were checked daily for bacterial growth for up to 72 hours. Bacterial organisms recovered were Gram-stained and sequenced as described above. The mean survival times of chicken embryos infected with different inocula were determined with Kaplan Meier survival curves.

3.2.7 Histopathology. One embryo each from eggs that died at 3, 4, and 5 days post-infection with the novel *Neisseria* isolate, and one viable control embryo were sent to the University of Minnesota Veterinary Diagnostic Laboratory for histopathologic examination.

3.3 Results

3.3.1 Samples Collected. We monitored a total of 237 white-front nests during the 2013 field season and identified at least one nonviable egg (addled or infertile) in 41 nests (17%). The majority of these nests contained a single addled egg (58.5%), whereas some nests contained a single infertile egg (9.8%), multiple addled eggs (9.8%), multiple infertile eggs (12.2%), or a combination of addled and infertile eggs (9.8%). In all, 36 addled eggs were collected from 28 nests and 17 infertile eggs were collected from 13 nests. The contents of addled eggs differed markedly from infertile eggs in visual appearance (Figure 3.1). Addled eggs showed failure of the perivitelline membrane resulting in loss of the distinction between yolk and albumin, and their texture varied from thin and serous to thick and caseous. The color of addled egg contents varied from yellow to green to grey in color while infertile eggs were characterized by a distinct yellow-to orange yolk and clear albumin.

3.3.2 Microbiology, PCR and Sequencing. Thirty of 36 aspirate samples from addled eggs were PCR positive for the bacterial 16S rRNA gene. Of those positives, 12 yielded sequence information and the remainder contained double peaks at most base pairs and were considered uninterpretable (Table 3.1). Six of 17 aspirate samples from infertile eggs were PCR positive for 16S rRNA but none yielded sequence information. Twenty-six of 36 addled eggs had at least one type of colony growth on blood and/or

chocolate agar and all 16S rRNA sequences generated from pure cultures were interpretable (Table 3.2). From these combined egg aspirate and culture PCRs, 23 *Neisseria*-like sequences were identified; 21 from pure cultures and 2 from egg aspirates (eggs that had no growth on blood or chocolate agar). *Macrococcus caseolyticus* was identified in 6 eggs and *Streptococcus uberis* and *Rothia nasimurium* were each identified in 4 eggs; *Shigella flexneri* and *Staphylococcus sciuri* were each identified in 2 eggs, and the remaining 5 bacterial species were each detected in a single egg. Most eggs from which multiple bacterial sequences were recovered after culture yielded uninterpretable sequences on initial aspirate PCR.

After development of a PCR assay to detect the cpn60 gene in our specific *Neisseria* isolate, the protocol was used on egg aspirate samples. All samples, in which we cultured and/or obtained a *Neisseria* sequence, were positive for *Neisseria* DNA using this new protocol. Four additional eggs from which we obtained different bacterial isolates also tested positive for *Neisseria* DNA. Two addled eggs with no bacterial growth and which tested negative for the bacterial 16S rRNA gene were additionally positive for *Neisseria* DNA using this new PCR protocol. Finally, four infertile eggs from which no growth and no bacterial 16S rDNA was identified did contain *Neisseria* DNA according to this new PCR protocol.

3.3.3 Cloacal and Nest Swabs. We developed a PCR to detect the *Neisseria* isolate found during the 2013 field season. A total of 91 swab samples were analyzed (Table 3.3). Of the 24 swabs collected prior to the onset of incubation (12 sets of an eggshell and a nest swab), in 4 sets both eggshell and nest material tested positive for DNA from our *Neisseria* isolate. However none of those was from a nest that later contained an addled egg from

which *Neisseria* was isolated. One preincubation nest swab was positive for *Neisseria* DNA, and that nest later contained an addled egg from which *Neisseria* was isolated; the eggshell swab from that sample was negative for *Neisseria* DNA.

Some samples collected during incubation were positive for *Neisseria* DNA as well. Of swabs collected from nests containing addled eggs (n=24), 20 were positive for *Neisseria* DNA. 15 of those nests contained eggs from which *Neisseria* was isolated. *Neisseria* DNA was also identified in the nest material of all 15 nests that were swabbed as controls (not containing an addled egg).

Twenty-eight cloacal swabs were assayed for *Neisseria* DNA, these samples were collected at hatch from white-fronted goose hens. Four swabs were positive for *Neisseria* DNA, but none were from hens that had been incubating an addled egg from which *Neisseria* was isolated. We did, however, identify *Neisseria*-addled eggs in the nests of two hens whose cloacal swabs tested negative for *Neisseria* DNA.

3.3.4 Morphology. Gram stain revealed Gram-negative cocci. Transmission electron microscopy of the *Neisseria*-like organism revealed the morphology of the culture as diplococcic (Figure 3.2) with a diameter of approximately 500nm.

3.3.5 *Neisseria* Phylogenetics. BLAST alignment results using partial 16S rRNA gene sequences identified *N. canis and N. animaloris* as the closest matches to our *Neisseria* isolate. The highest identity score for any of our isolates is 97%. A neighbor-joining tree including our 23 *Neisseria-like* isolates and other *Neisseriales* sequences from GenBank is shown in Figure 3.3 and shows that our isolates cluster together and show some genetic variation.

Given the frequency of the occurrence of *Neisseria*-like bacteria in addled eggs (isolated and/or sequenced from 23 of 36 addled eggs) and the unclear species distinction of these bacteria, we placed additional focus on characterizing this organism. We sequenced the cpn60 gene from all putative *Neisseria* isolates, and obtained full-length 16S rRNA gene sequences from a subset of our isolates.

The phylogenetic tree derived from cpn60 sequences obtained from our isolates and from publicly available sequences (Genbank) (Figure 3.3) shows a cluster of these isolates with some variability, though this tree locates our isolates at about equal distance to *N. wadsworthii* and *N. canis*.

Finally, a tree constructed from full length (1298-1448 bp) 16S rRNA gene sequences of our isolates and others in the class betaproteobacteria clearly show our isolates in a distinct cluster, but nearest to *Neisseria canis* and others in the family *Neisseriales* (Figure 3.4). Additionally, all of our full-length 16S rRNA gene sequences had ≤97% identity scores. Bacteria sharing ≤97% 16S rRNA base pairs generally are considered to be different species (31).

3.3.6 Embryonated Egg Infections. The majority of embryos from eggs infected with the *Neisseria* isolates died by day 7 post infection although there was some indication that survival varied by inoculation dose; all embryos inoculated with greater than 10,000 colony forming units (cfu) died by day 5, while 10% of embryos inoculated with 1,000 cfu survived 7 days post-infection, and 30% of eggs infected with 100 cfu survived the 7-day trial (Figure 3.5). All eggs inoculated with *S. uberis* (10^0-10^4 CFU) died by day 4 post-infection. All eggs inoculated with 10^6 CFU of *R. nasimurium* died by day 4; 10^5 and 10^6 CFU died by day 5, 10^3 CFU died by day 4, and 1 egg inoculated with 10^2 *R. nasiumrium* survived

until 7 days post-infection. All eggs inoculated with 10^6 CFU of *M. caseolyticus* died by day 5. Eggs inoculated with all other dilutions of *M. caseolyticus* (10^5-10^2) died by day 7 post-infection. All control eggs survived inoculation with PBS.

Histopathology of embryos inoculated with putative *Neisseria* showed marked underdevelopment, tissue degeneration and necrosis. Organ, tissue, and cellular details were obscured by cellular infiltrates. Bacterial colonization with Gram-negative cocci was present in 2 of 3 embryos.

3.3.7 Sequence Accession Numbers. The sequence data from this study have been deposited in GenBank. Putative *Neisseria* isolate 16S rRNA sequences are under accession numbers KF995745-KF995749, KF999688-KF999690, KJ596479-KJ596481, KF999694-KF999695, KJ596482, and KF999697-KF999705. Putative *Neisseria* isolate cpn60 sequences are under accession numbers KJ508837-508856. Sequences obtained using apparent *Neisseria*-specific PCR (cpn60) are under accession numbers KM233718-KM233764. Other isolate sequences are under accession numbers KJ652676-KJ652699. *Neisseria canis* cpn60 sequence is GenBank accession number KJ872773.

3.4 Discussion

Using culture and PCR, we detected a total of 11 species of bacteria in addled white-fronted goose eggs. The most prevalent was a single species in the *Neisseria* genus that was isolated from 21 addled eggs, and 16S rRNA gene sequences corresponding to this *Neisseria* isolate were recovered from 2 additional eggs that had no apparent bacterial growth. Using a PCR protocol developed specifically to detect this *Neisseria* isolate, we detected DNA in an

additional 6 addled eggs and in 4 infertile eggs. We also detected *Macrococcus, Streptococcus, Staphylococcus,* and *Rothia* in our samples.

Bacteria from eggshells and egg contents have been isolated from a range of other avian species. Ruiz-de-Casteñada (10) described microorganisms on the eggshells of flycatchers in a temperate environment. They did not detect any Gram-negative cocci on eggshells, but did find *Serratia fonticola*, which was isolated from the contents of one egg in our study (Table 3.2). Another study focused on nonviable raptor eggs in Canada and found that most had heavy bacterial growth; *E. coli, Streptococcus* and other organisms were isolated from the contents of addled eggs (32). Finally, Pinowski (33) found that 70% of sparrow (*Passer domesticus* and *P. montanus*) eggs that did not hatch contained bacteria, including *E. coli*, *Staphylococcus* spp., *Streptococcus* spp., and *Serratia fonticola*. Thus, the presence of *Streptococcus* and *Staphylococcus* bacteria in addled eggs appears to be common. It is perhaps unusual that we did not identify *E. coli* bacteria in our samples given that it has been found in all previous studies. Our identification of *Neisseria* as the predominant bacteria isolated from addled eggs appears to be novel.

While *Neisseria* has not been associated with egg contents in wild bird populations, previous reports of "goose gonorrhea" in domestic geese in Hungary may be relevant (34, 35). These reports describe a disease of the phallus and cloaca; pathology includes swelling and mucosal reddening, necrotic inflammation, prolapse, and sometimes partial loss of the phallus. Afflicted domestic goose flocks had decreased feed intake, decreased egg production, and increased sterility (34). Pataky et al. (35) described the organism associated with goose gonorrhea as a Gram-negative coffee-bean shaped mono- or

diplococci that is 0.5 μm in diameter placed the organism in the *Neisseria* genus. Further, three isolates whose DNA sequences most closely matched *N. musosa* (100%), *N. canis* (96%), and *N. meningitidis* (96%), were isolated from duck feces in New Zealand (36).

We did not isolate bacteria or amplify the bacterial ribosomal RNA gene from all collected addled eggs. Eight eggs confirmed addled by visual inspection in the laboratory did not demonstrate any bacterial growth, and were either PCR negative for 16S rRNA, or had weak uninterpretable sequences. Two of those eggs did contain detectable *Neisseria* DNA as evidenced by a PCR developed to detect our isolate. Our incapacity to detect all *Neisseria* in eggs initially may be due to the presence of other bacteria in some of those samples that may have overgrown the *Neisseria* in culture, or the presence of *Neisseria* at very low levels, or the possibility that *Neisseria* had died during transport from the field. Additionally, DNA may have been present at very low levels and may have been below the detection limit of our broad-based 16S rRNA gene PCR protocol.

Despite the increased detection of *Neisseria* with our new PCR protocol, not all addled eggs yielded bacterial DNA or bacterial growth. This implies that other causes may be responsible for some of our documented embryo mortality, or that our diagnostic sensitivity (culture and PCR) was less than 100%. It is plausible that only one compartment of an egg is infected (yolk, albumin, embryo), and due to the limited volume aspirated, bacteria may not have been sampled. For example, Cook et al. (4) isolated bacteria from different egg compartments, and not all species were present in the same compartments. Thus, microbial infection may be the primary cause of embryonic mortality, but we may

have underestimated the prevalence rate for some of the bacteria we identified and other taxa of bacteria may be present in our samples but not detected via our methods.

Most studies of avian embryo mortality from microbial pathogens have focused on trans-shell infection as the primary route of transmission (4-7). However, it has also been shown that some species of bacteria (e.g., *Salmonella*, *Campylobacter*, *Mycoplasma*) may infect eggs prior to laying via direct contamination of reproductive organs (15, 16, 37). We attempted to identify the source and possible route of transmission of the most commonly isolated bacteria (a *Neisseria* species) by analyzing swab samples from eggshells, nest contents, and cloacae of nesting females. Our results indicate that the *Neisseria* isolate found in most addled eggs is widespread in the nest environment (i.e. nest materials and egg shells) and infection of eggs may occur post laying. However, we also detected *Neisseria* in some cloacal swab samples implying that eggs may have been infected prior to laying. In two instances, cloacal swabs were negative for *Neisseria* DNA, but *Neisseria*-addled eggs had been identified in their nests earlier in the nesting season. This may mean that hens are infected, pass the bacteria to their eggs, and can clear the infection themselves, or that the bacteria is originating higher in the reproductive tract.

Given these results, our data are inclusive in terms of identifying the potential source of the *Neisseria* we isolated from eggs, and it is possible that multiple modes of transmission are occurring (as occurs with *Salmonella*). Further, not all eggs associated with contaminated nests or females were addled, implying that transfer to eggs is not ubiquitous or that eggs tend to be infected with relatively low quantities of bacteria. Based on our inoculation study, the LD50 for our *Neisseria* isolate is low (less than 100 CFUs), but some eggs infected at low doses did survive.

Sequence data suggest that the *Neisseria* species isolated in this study has not previously been described. Phylogenetic analysis using 16S rRNA and cpn60 gene sequences suggest that it belongs to the *Neisseria* genus, but species distinction is unclear. None of our full-length 16S rRNA gene sequences are more than 97% similar to existing sequences, which traditionally has been the cutoff for the species level (31). Therefore, our isolates appear to be distinct from other described species. We also detected some variability between the isolates from addled eggs, meaning that this is not recent expansion of a single clone. This pattern, along with our alignment scores, suggests that the *Neisseria* we have isolated may be a previously undescribed species and deserves further investigation.

The family *Neisseriales* currently contains 32 genera that occupy a wide range of habitats including oral, gastrointestinal, and reproductive tracts of many species (reviewed in 38). Two well-known human pathogens do exist in the genus (*N. gonorrhoeae* and *N. meningitidis*), and other species are occasionally isolated from a variety of sources (39). Organisms that are closely related to our *Neisseria* isolate tend to be oral and gastrointestinal commensals, but are sometimes associated with disease. *Neisseria canis* is often associated with periodontal disease in dogs (40). *Neisseria weaveri* and *N. animaloris* (and other species) are sometimes isolated from animal bite wounds (41, 42). A species with a similar sequence to our isolate was isolated from the liver of a sheldrake *(Tadorna tadorna)* that was found dead in China (43). The probable source of the *Neisseria* isolates we identified is unclear, but other species in the genus tend to be oral and gastrointestinal tract commensals, so it is likely that this isolate is originating from the hen in some way.

In addition to our commonly identified *Neisseria* isolate, three other species of bacteria were isolated from >3 addled eggs. The first, *Macrococcus caseolyticus*, (isolated from 6 eggs) is typically found in cow's milk and is generally not considered a pathogen (44). There are no reports of any *Macrococcus* sp. in bird eggs. The second, *Streptococcus uberis*, (isolated from 4 eggs) is a well-known cause of mastitis in cattle (45), but has not previously been isolated from bird eggs. However, there are reports of other *Streptococci* being isolated from eggs (4, 32). Finally, *Rothia nasimurium* (isolated from 4 eggs) is most commonly isolated from the upper respiratory tract of pigs and mice (46). While other species in the *Rothia* genus occasionally cause disease in humans (47), there are no reports of any *Rothia* species being isolated from bird eggs. All of these bacterial isolates are or are related to organisms that are most commonly commensals or opportunistic pathogens (except *S. uberis*), and are typically found in animals. Accordingly we suspect that the ultimate source for all these bacteria is the birds themselves. Given that white-fronted geese spend the winter months in the southern United States, it is plausible that these isolates originated there, perhaps from contact with domesticated animals.

Our results demonstrate that embryo mortality in greater white-fronted goose eggs on the Arctic Coastal Plain of Alaska is likely caused by bacterial infection. This study provides the first evidence of microbial-induced hatching failure in wild geese and in an Arctic ecosystem. We detected a potentially novel species of *Neisseria* in numerous addled eggs that were previously observed in normal embryo development, and in no infertile eggs. Further, inoculations of bacterial isolates into developing chicken eggs provided clear evidence of this organism's pathogenicity. We also isolated and demonstrated mortality potential of three other bacterial species: *Macrococcus caseolyticus*, *Streptococcus uberis*,

and *Rothia nasimurium*. Future research should focus on source and route of infection, possible reservoirs, and geographic extent of *Neisseria* and other bacterial sources of embryo mortality.

3.5 Acknowledgments

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We thank the field crew at Point Lonely (Mike Johnson, Tim Spivey, Curtis Twellmann) for help collecting samples and Ryan Adam for technical support in the lab. Paul Flint provided valuable assistance with project design. Transmission electron micrographs were performed at the Advanced Instrumentation Laboratory at the University of Alaska Fairbanks.

Table 3.1: Partial 16S ribosomal RNA gene PCR results from egg aspirate samples. Primers F2C and R2C were used for PCR; primer R1 was used for sequencing. Sequences with more than one peak at each base pair were considered uninterpretable and likely resulted from more than one species of bacteria being present in each sample.

n	Status	PCR result	BLASTn Match[a]	rdp Match[b]
6	Addled	+	*Neisseria animaloris* or *N. canis* (95-96%)	Bacterium "New Zealand A" (99-100%)
5	Addled	+	*N. animaloris* (96-97%)	Bacterium "New Zealand A" (99-100%)
1	Addled	+	*Helcococcus ovis* (91%)	*H. ovis* (68%)
18	Addled	+	Uninterpretable	Uninterpretable
6	Addled	-	-	-
6	Infertile	+	Uninterpretable	Uninterpretable
11	Infertile	-	-	-

a. National Center for Biotechnology Information nucleotide BLAST (Basic Local Alignment Search Tool; BLASTn), http://blast.ncbi.nlm.nih.gov/Blast.cgi

b. Ribosomal database project (rdp) seq match, http://rdp.cme.msu.edu/seqmatch.

Table 3.2: Pure culture partial 16S rRNA gene BLASTn sequence matches for all isolates obtained from addled eggs. Primers F2C and R2C were used for PCR; primer R1 was used for sequencing.

n	BLASTn[a] Match Isolate 1	BLASTn Match Isolate 2	BLASTn Match Isolate 3
5	*Neisseria animaloris* or *canis* (95-96%)		
3	*N. animaloris* (96-97%)		
3	*N. animaloris* (96-97%)	*Macrococcus caseolyticus* (98%)	
2	*N. animaloris* (96-97%)	*Streptococcus uberis* (99-100%)	
2	*N. animaloris* (97%)	*Rothia nasimurium* (98-99%)	
1	*N. animaloris* or *canis* (96%)	*R. nasimurium* (97%)	*Ottowia thioxydans* (96%)
1	*N. animaloris* or *canis* (97%)	*R. nasimurium* (97%)	*Moraxella cuniculi* (98%)
1	*N. animaloris* or *canis* (96%)	*M. caseolyticus* (97%)	
1	*N. animaloris* (96%)	*M. caseolyticus* (98%)	*Staphylococcus sciuri* (99%)
1	*N. animaloris* (96%)	*Paracoccus yeei* (98%)	
1	*N. animaloris* or *canis* (96%)	*Stenotrophomonas rhizophila* (100%)	
1	*S. uberis* (100%)	*S. sciuri* or *vitulinus* (99%)	
1	*M. caseolyticus* (98%)	*Serratia fonticola* (99%)	
1	*S. uberis* (100%)	*Shigella flexneri* (99%)	
1	*S. flexneri* (99%)		
11	No growth		

[a]. National Center for Biotechnology Information nucleotide BLAST (Basic Local Alignment Search Tool; BLASTn), http://blast.ncbi.nlm.nih.gov/Blast.cgi

Table 3.3: Greater white-fronted goose (*Anser albifrons*) bacteriologic swab samples collected during lay (preincubation), incubation, and at hatch at Point Lonely, Alaska, in 2013. The PCR assay conducted amplifies a 304 bp segment of the chaperonin 60 gene and is specific for our *Neisseria* isolate. Addled with *Neisseria* indicates the number of eggs from that subset that were identified as addled, and where our *Neisseria* isolate was identified in egg contents.

Preincubation Samples			
	Nest/egg combinations (n=12 nests, 12 eggs)	n	Addled with *Neisseria*
	Egg and Nest PCR +	4	0
	Egg - and Nest +	1	1
	Egg and Nest PCR -	7	0
Incubation Samples (egg and nest material)			
	Swabbed with addled egg in nest[a] (n=24)		
	PCR +	20	15[b]
	PCR -	4	2
	Swabbed as control (no addled egg, n=15)		
	PCR +	15	0
	PCR -	0	0
Hatch Samples			
	Cloacal swabs[c] (n=28)		
	PCR +	4	0
	PCR -	24	2

[a]. Two nests had been swabbed with preincubation samples

[b]. One nest had been swabbed with preincubation samples

[c]. Two cloacae were from nests that had been swabbed with preincubation samples

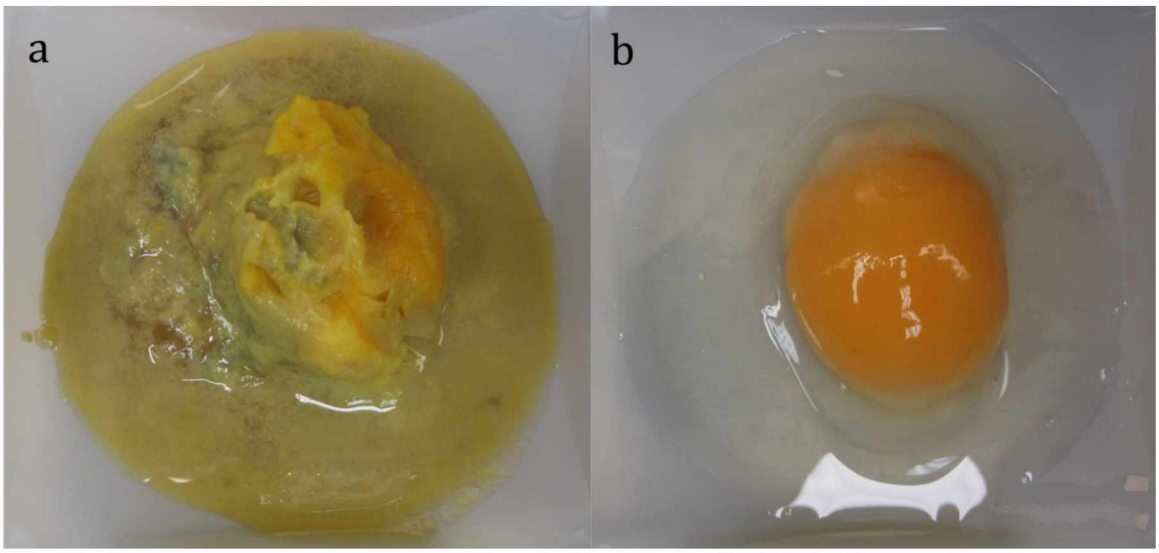


Figure 3.1: An example of the contents of an addled egg (a) compared to an infertile egg (b). Eggs were collected at Point Lonely, Alaska, during the summer of 2013.

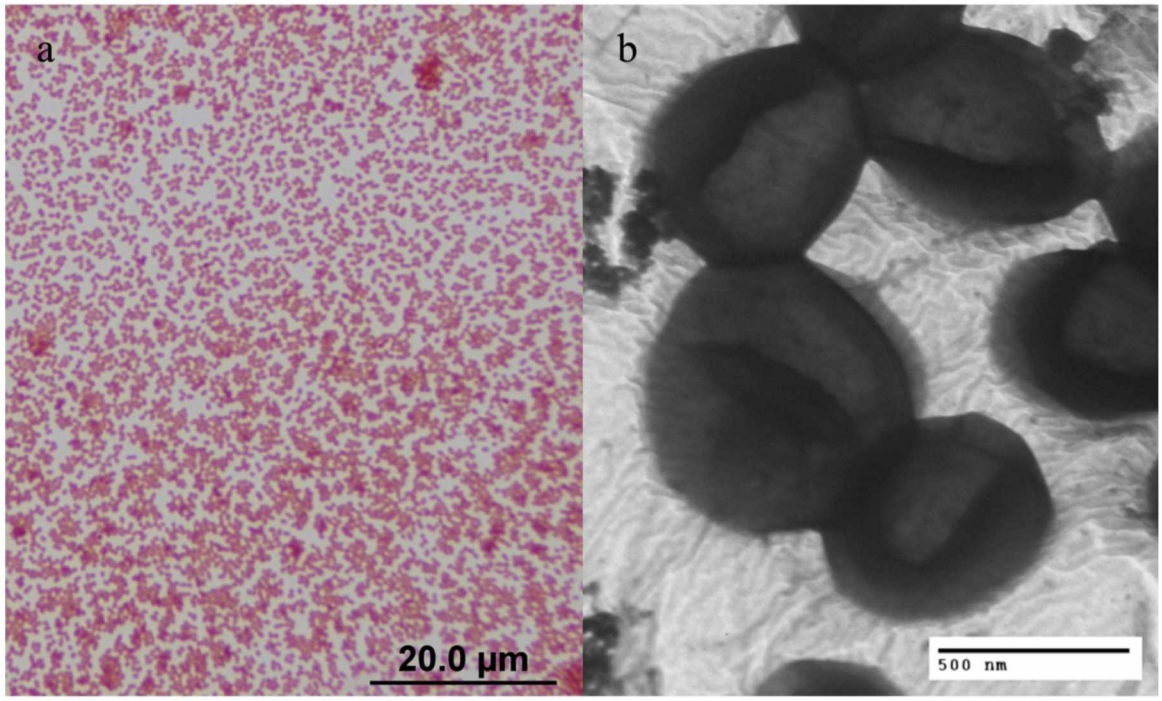


Figure 3.2: Gram stain performed with standard techniques (a) and showing small Gram-negative diplococci. Transmission electron micrograph (b) of one of our *Neisseria* isolates showing approximately 500 nm diplococcic organisms with spherical to coffee bean shapes.

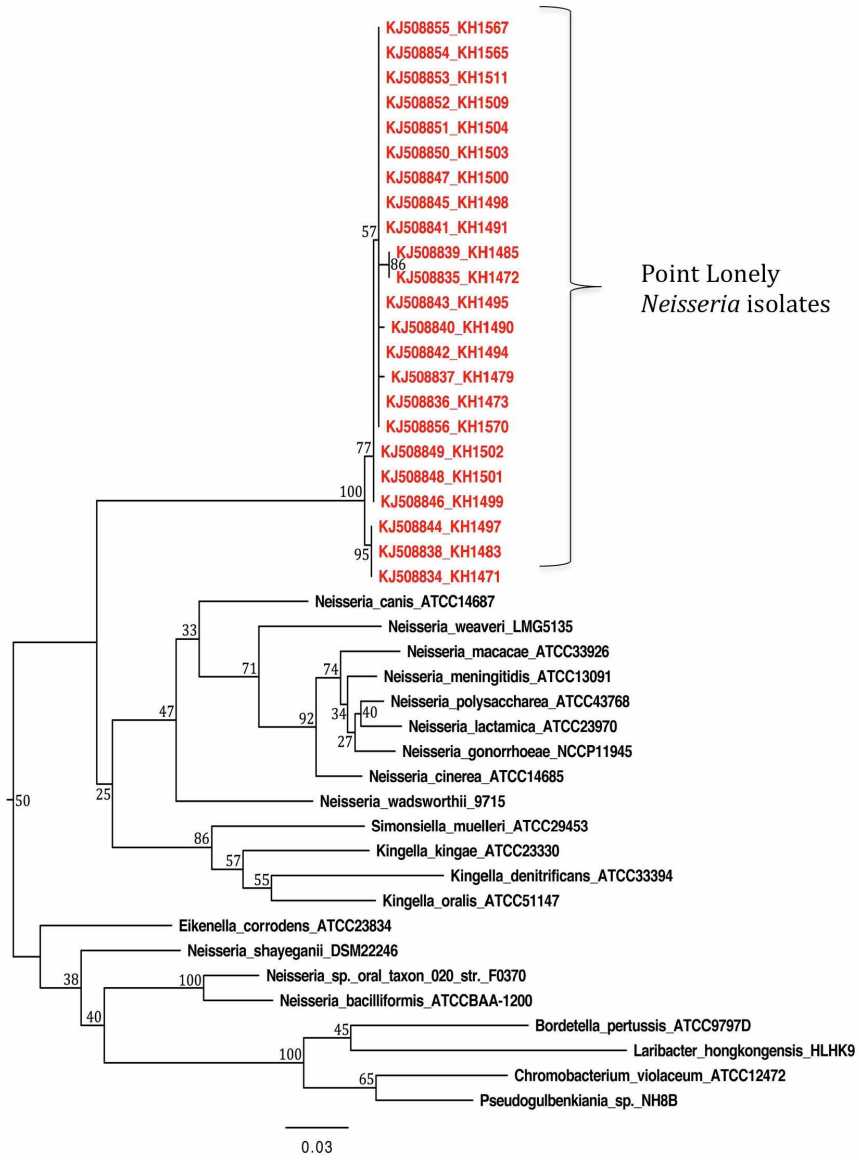


Figure 3.3: Neighbor-joining tree based on 406 bp of the cpn60 gene. Bootstrap values are shown at nodes and are based on 2000 replicates. Point Lonely, Alaska, *Neisseria* isolates are shown in red and are denoted by both GenBank accession number (begins with KJ) and our sample number (begins with KH). Additional sequences from the family *Neisseriales* were obtained from the cpn60 database (http://www.cpndb.ca/cpnDB/home.php)

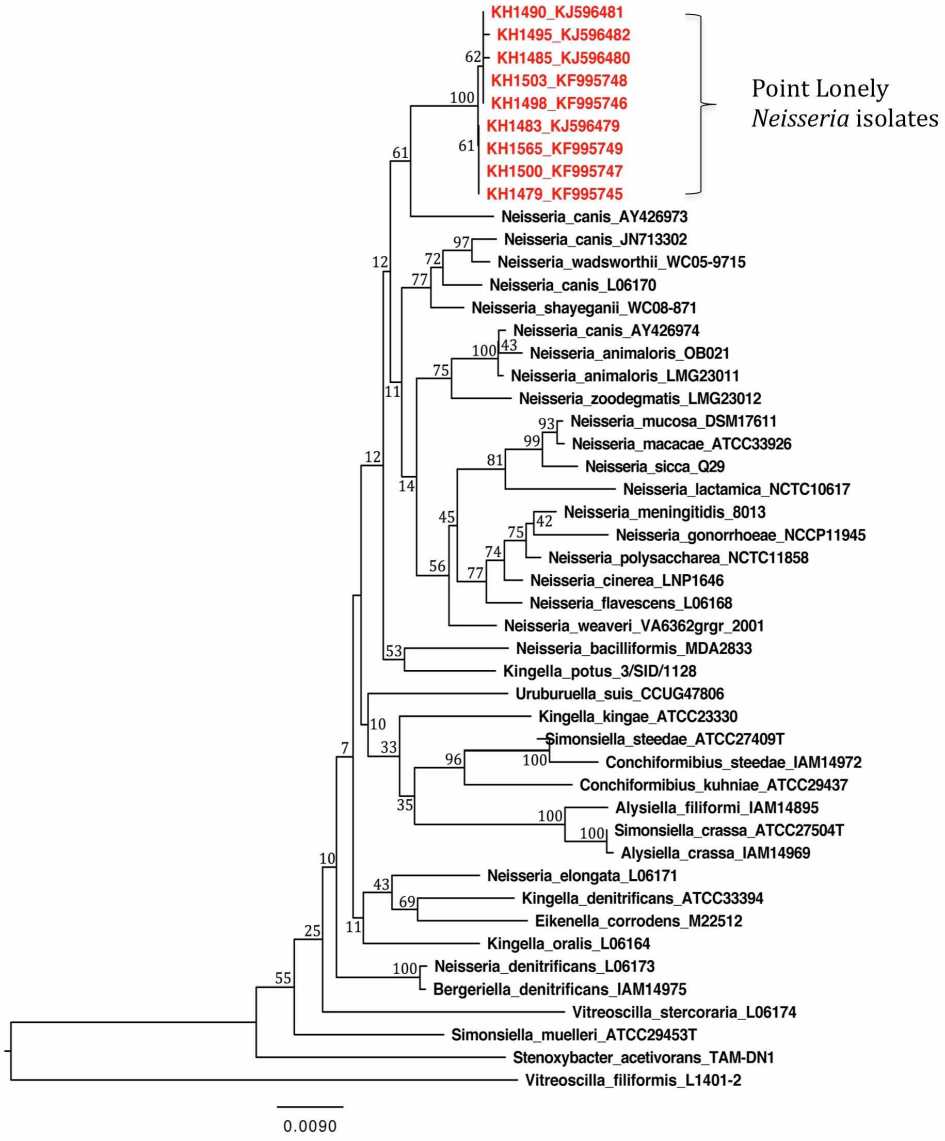
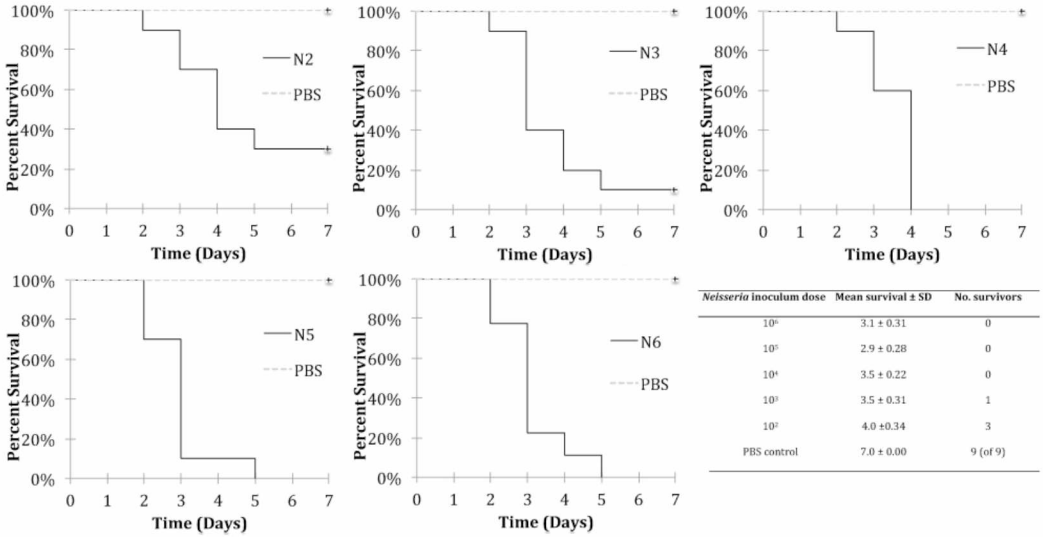


Figure 3.4: Neighbor-joining tree based on full (1243 bp) 16S ribosomal RNA gene sequences. Bootstrap values are shown at nodes and are based on 2000 replicates. Point Lonely, Alaska, *Neisseria* isolates are shown in red and are denoted by both GenBank accession number (begins with KF) and our sample number (begins with KH). Additional sequences from the family *Neisseriales* were obtained from the ribosomal database project (http://rdp.cme.msu.edu/).



Neisseria inoculum dose	Mean survival ± SD	No. survivors
10^6	3.1 ± 0.31	0
10^5	2.9 ± 0.28	0
10^4	3.5 ± 0.22	0
10^3	3.5 ± 0.31	1
10^2	4.0 ±0.34	3
PBS control	7.0 ± 0.00	9 (of 9)

Figure 3.5: Kaplan-Meier survival curves for embryonated chicken eggs infected with two different strains of our *Neisseria*-like bacterium. Percent survival is shown on the Y-axis, days post-infection on the X-axis. Five eggs were inoculated with each dilution (10^6-10^2 bacteria per egg) of each strain of *Neisseria*.

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Chapter 4

Use of cellulose filter paper to quantify whole blood mercury in two marine mammals: Validation study[1]

Abstract

Whole blood is commonly used to assess mercury (Hg) exposure in mammals, but handling and shipping samples collected in remote areas can be difficult. We describe and validate use of cellulose filter papers (FP) for quantifying whole blood total Hg concentration. Advantec Nobuto® FP were soaked with bottlenose dolphin (*Tursiops truncatus*) or harbor seal (*Phoca vitulina*) whole blood (collected between March and July 2012) then air-dried. Untreated blood-soaked FP were analyzed or were eluted with phosphate buffered saline (PBS) and the eluate and PBS treated FP Hg concentrations were determined. Total Hg from dried blood-soaked FP, post-elution FP, and PBS-based eluate were compared to total Hg concentrations from whole blood. Recovery (on a concentration basis) for soaked FP relative to whole blood was 0.89±0.15, for post-elution FP was 0.86±0.13, and for eluate (with a correction factor applied) was 0.96±0.23. Least squares linear regressions were fit for soaked papers ($y=1.15x$, $R^2=0.97$), post-elution filter papers ($y=1.22x$, $R^2=0.95$), and for eluate with a correction factor applied ($y=0.91x+0.03$, $R^2=0.97$) as compared to whole blood. These data show that FP technology can have a valuable role in monitoring blood Hg concentrations in wildlife populations and FPs have the advantage of being easy to use, store, and transport as compared to whole blood.

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[1] Hansen CM, Hueffer K, Gulland F, Wells Randall S, Balmer BC, Castellini J, O'Hara T. 2014. Use of cellulose filter paper to quantify whole blood mercury in two marine mammals: Validation study. *J Wildl Dis* 50(2):271-278.

4.1 Introduction

Mercury (Hg) is a nonessential element that occurs naturally in the environment. Mercury is released into the atmosphere via events such as volcanic eruptions and forest fires. Since the industrial revolution, anthropogenic releases of Hg into the environment have increased, mostly through the burning of fossil fuels and via the mining industry, and may occur at concentrations of concern to health in some biota (e.g. Dietz et al., 2009, 2013). Following deposition of atmospheric Hg into marine and freshwater systems, microbial activity (largely sulfate reducing bacteria) can transform Hg to the highly bioavailable and toxic monomethylmercury ($MeHg^+$) (Fitzgerald et al., 2007; Parks et al., 2013). Monomethylmercury can bioaccumulate and biomagnify with trophic levels (Coelho et al., 2013), reaching particularly high levels in numerous fish species and piscivores (Castoldi et al., 2001; Lemes et al., 2011; Castellini et al., 2012).

Following ingestion, $MeHg^+$ is absorbed via intestinal epithelium passively and via active uptake (Leaner and Mason, 2002), and is nearly completely absorbed. Crossing the intestinal epithelium, $MeHg^+$ enters the blood where 99% binds to thiol groups, the remaining 1% is transported to organs via binding to diffusible low molecular weight thiols (Rooney, 2007). Hence blood is the route of exposure (and distribution) for most target organs (i.e. the central nervous system) and is a reliable indicator of recent $MeHg^+$ exposure (Risher and Amler, 2005).

A key target organ for $MeHg^+$ toxicity is the central nervous system as $MeHg^+$ crosses the blood brain barrier via an amino acid transporter and accumulates in nervous tissue (Kerper et al., 1992; Caito et al., 2013). Clinical signs of acute toxicity include

proprioceptive deficits, abnormal postures, blindness, anorexia, coma, and death (Ekino et al., 2007). High levels of $MeHg^+$ have been shown to impair components of the nervous system (Basu et al., 2006, 2007b). There is concern that, particularly in fish-eating wildlife, chronic exposure to $MeHg^+$ can result in poor reproductive success (Basu et al., 2007a). There is also concern that Hg levels in wildlife, and in humans that subsist on wildlife (particularly in higher latitudes) may be reaching concentrations that can have impacts on behavior and health (Grandjean et al., 1997; Castoldi et al., 2001; Oken et al., 2005; Holmes et al., 2008; Basu et al., 2009; Bocharova et al., 2013); especially for the fetus and neonate (Castellini et al., 2012; Rea et al., 2013).

Whole blood is commonly used to assess Hg exposure (Brookens et al., 2007; Knott et al., 2011). Blood is relatively easy to access (relative to target tissues such as the kidney and nervous system), is commonly collected by biologists, veterinarians, and others who work with wildlife in the field, and is a good tissue for determining Hg status in wild animal populations. Hair is easily accessible and used for monitoring Hg status in wildlife and is more useful for long-term (chronic) mercury assessment as hair Hg concentration represents the average concentration of Hg in circulating blood (Budtz-Jorgensen et al., 2004).

There are long-term mercury monitoring programs in place for wildlife, particularly fish (Great Lakes Fish Monitoring and Surveillance Program, http://www.epa.gov/grtlakes/monitoring/fish/index.html), and monitoring sometimes follows contamination events (Alvarez et al., 2013). Monitoring programs for humans exist as well (Alaska Hair Mercury Biomonitoring Program, State of Alaska Epidemiology *Bulletin* 2013; Alaska Native Maternal Organics Study (MOM Study) operated by the Alaska Native

Tribal Health Consortium (ANTHC). However, blood is less commonly used for biomonitoring due to relative difficulty (compared to hair) with collection, storage, and transport. Collection in the field can be particularly problematic, especially in remote locations with limited processing and preservation capabilities. The development of a blood sampling regime that can be easily used in the field by scientists, hunters, fishermen, or other trained people would facilitate clinical, research, and biomonitoring efforts. Here we describe the use of cellulose filter papers for collection of blood in the field and subsequent analysis of total Hg concentration in various filter paper matrices in comparison with whole blood collected in standard blood collection tubes.

4.2 Materials and Methods

4.2.1 Filter Paper and Samples

Advantec Nobuto® cellulose filter papers (Dublin, CA, USA) were purchased from Cole-Parmer (Vernon Hills, IL, USA) and were used for all investigations (fig 4.1). FP were either used singly or were fashioned into combs of 5 or 6 papers for use in the field (Curry et al., 2011). Whole blood (WB) samples were collected between March and July 2012 from wild harbor seals (*Phoca vitulina*) brought to The Marine Mammal Center (Sausalito, CA, USA, MMPA permit no. 932-1905/MA-009526) for rehabilitation and from long-term resident bottlenose dolphins (*Tursiops truncatus*) live captured, sampled, and released following health assessments in Sarasota Bay, FL, during May and July 2012, by staff from the Chicago Zoological Society (Wells et al. 2004; NMFS Scientific Research Permit No.

15543, IACUC 11-09-RW1). Blood samples were collected into BD (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) Vacutainers™ containing K_2EDTA as an anticoagulant. The narrow absorbing ends of 10 – 12 filter papers were soaked in whole blood (approximately 100 μL/strip) following collection and FP were air dried overnight. The fluid sample of whole blood was stored frozen (-20° C). For each individual animal, dried filter paper samples were shipped overnight at room temperature in a sealed plastic bag with paper towels layered between each sample and blood samples were shipped accompanied by freezer packs, to the Wildlife Toxicology Laboratory (WTL) at the University of Alaska Fairbanks (Fairbanks, AK, USA).

4.2.2 Sample Preparation

Prior to chemical analysis, control (n = 10, no blood) and blood-soaked FP were freeze-dried for 48 hours in a Labconco FreeZone 6 Plus freeze dryer (Kansas City, MO, USA). The narrow absorbing ends of FP_C (control) and FP_{WB} (soaked, whole blood) were cut (using a disposable razor blade) at the junction of the narrow and wide ends (Fig. 4.1) and weighed to determine the dry mass of blood on each paper (Mass WB = Mass FP_{WB} – Average Mass FP_C). All 60 FP_C and three FP_{WB} from each individual animal sample set were analyzed directly for total mercury concentration ([THg]). The [THg] was calculated based on the mass of mercury (ng) and mass of blood (~100mg) on each strip. Three more FP_{WB} from each individual animal sample set were separately eluted according to the protocol developed by Curry et al. (2011). Each FP_{WB} was cut into 5-7 pieces into a 2 mL pre-weighed cryogenic tube (Thermo Scientific, Waltham, MA, USA) using stainless steel iris

scissors. Each strip was then covered with 400 μL of phosphate buffered saline (PBS; Gibco, Carlsbad, CA, USA) with 1% penicillin-streptomycin (Gibco, Carlsbad, CA, USA). Each cryogenic tube was agitated to ensure filter papers were soaked, and were eluted overnight (16 hours) at 4° C.

After 16 hours, approximately 200 μL of eluate (E) were removed from each cryovial using a micropipettor. Eluate was transferred to a 1.5 mL microcentrifuge tube (Fisher Scientific, Waltham, MA, USA) and held at -50°C until analysis. Post-elution filter papers (including ~ 200 μl of remaining eluting buffer) were again freeze dried for 48 hours. Following drying, each cryovial (containing post-elution FP pieces) was weighed to determine the final weight of the post-elution paper (FP_E).

4.2.3 Mercury Analysis

All samples (WB, FP_C, FP_{WB}, FP_E, and E) were analyzed for [THg] on a Milestone DMA-80 Direct Mercury Analyzer (Milestone Inc., Shelton, CT, USA) (US EPA method 7473) using a 16 point calibration curve from 0.25 ng to 400 ng similar to Knott et al. (2011). Samples were analyzed in triplicate when possible (i.e. when there were enough filter papers for each sample). Single filter papers (for FP_E a single filter paper included 5-7 cut pieces) were analyzed in nickel sample boats and whole blood (~100 μl) and eluates (100 μl) were analyzed in quartz sample boats. The detection limit using this method was 5 ng/g for 100 μl of blood or eluate and 2.5 ng/g for 200μl of eluate.

Quality control included a 10 ng (1 ng/g) liquid calibration standard (Perkin Elmer, Waltham, MA, USA, item #, N9300133), and DORM-3 (National Research Council Canada,

Ottawa, ON, Canada) and DOLT-4 (National Research Council Canada, Ottawa, ON, Canada) certified standards analyzed in triplicate in each DMA80 run. Recoveries were 94.6±0.05% ng/g (10 ng), 102.2±4.4% (DORM-3, reference range 0.382±0.060 mg/kg), and 100.1±6.8% (DOLT-4, reference range 2.58±0.22 mg/kg).

4.2.4 Calculations and Statistics

Data were managed in Microsoft Excel, and statistics were performed using the program R (http://www.r-project.org/, version 2.14.12, downloaded 2/29/2012). Least squares linear regressions were fit to FP_{WB}, FP_{E}, and E compared to WB. Confidence intervals (95%) for slopes were constructed, and slopes were compared to a test value of 1 using R package smatr (http://cran.r-project/web/packages/smatr/index.html). Student's paired t-tests were used to compare [THg] means of FP_{WB}, FP_{E} , and E to WB.

Whole blood data were converted to a dry weight basis using the proportion of dry matter in WB. For some calculations and statistics wet weight concentrations are reported, for others, dry weight concentrations. To determine the dry weight of blood from each species 100 μL of whole blood from each animal was weighed, freeze dried for 48 hours, and re-weighed. The dry blood weights were 24.9% ± 1.8 SD for harbor seals and 20.5 ± 0.7% for bottlenose dolphins.

A correction factor was applied to eluate samples to estimate the original WB (wet) [THg] (Fig. 4.2). The elution process involves adding 400 μL of PBS (~0.400 g) to strips (FP_{WB}) containing dried components (0.2 – 0.25 g) from approximately 100 μL (~0.100 g) of blood. Therefore a correction factor (CF) was estimated for each sample as follows:

$$CF = \text{Mass of E/Mass of } WB_{wet}$$

$$= (0.400\text{ g} + \text{Weight (in g) of dry blood on } FP_{WB})/0.100\text{ g}.$$

This correction factor was then applied to eluate [THg]:

$$E_{CF} = E*CF \approx WB\text{ (wet)}$$

This correction factor result was compared to the original WB (wet) [THg].

4.3 Results

The average weight of the narrow part (Fig. 4.1) of FP_C is 0.0466 ± 0.002 g (n = 10). Single control filter papers not soaked with blood were below the detection limit of the DMA-80 (0.5 ng/FP, n = 10). Mean [THg] values (on a concentration basis) for WB, FP_{WB}, FP_E, and E are summarized in Table 4.1. FP_{WB}, FP_E, and E [THg] relative to [THg] in WB in matched samples is summarized in Figure 4.3. For dolphins, the relative proportion of [THg] in FP_{WB} and FP_E compared to whole blood is 0.87 ± 0.08 and 0.82 ± 0.13, respectively. For harbor seals, the relative proportion of [THg] in FP_{WB} and FP_E compared to whole blood was more variable at 0.95 ± 0.42 and 0.92 ± 0.32, respectively. The mean difference between the proportion of [THg] FP_{WB} compared to WB is 0.04 ($p<0.001$), between FP_E and WB is 0.05 ($p<0.001$), and there is no mean significant difference between E_D WB ($p=0.4$) (paired t-tests).

Figure 4.4 shows [THg] WB regressed on FP_{WB}, FP_E, and E values. Data for WB, FP_{WB}, FP_E, and E are presented on a wet weight basis. The R^2 for blood-soaked filter papers is 0.97, for post-elution filter papers is 0.95, and for eluate (with correction factor applied) is 0.97. A 95% confidence interval for the slope is 1.12 – 1.19 for WB regressed on FP_{WB},

1.18-1.32 for WB regressed on FP_E, and 0.89-0.97 for WB regressed on E. Tests for each slope (H_O: slope =1 or y=x) indicates $p < 0.01$ for each regression (Fig 4.3).

4.4 Discussion

We used blood soaked FP samples to assess mercury concentrations in the blood of bottlenose dolphins and harbor seals. The values for whole blood total mercury for bottlenose dolphins and harbor seals from our study populations (Table 4.1) are within the ranges previously reported (Brookens et al., 2007; Woshner et al., 2008).

Advantec Nobuto filter paper strips are uniform in size and weight (0.0446±0.002 g), and their [THg] is below the detection limit of a DMA80 (<0.5 ng). Our data support that cellulose FPs soaked in whole blood and air-dried are an accurate and reproducible way to quantify whole blood [THg] for some mammals. Overall recoveries on a concentration basis are very high, ranging from 82-95%, when compared to whole blood concentration for FP_{WB}, FP_E, and E_{CF} (Fig 4.3). Additionally, with R^2 values of 0.97, 0.95, and 0.97 respectively for FP_{WB}, FP_E, and E_{CF} (Fig 4.4), whole blood mercury concentration can be easily estimated from dried and/or eluted samples, provided [THg] is high enough to be detected.

This technique promises to be valuable to scientists, wildlife managers, veterinarians, and others needing a simple, inexpensive, and highly effective method for collecting blood samples for mercury analysis in combination with other assays. Perhaps even more importantly, these filter papers could be distributed to hunters and used in the field to increase the scope of wildlife monitoring programs. Programs aimed at developing community-based wildlife health monitoring programs exist (Brook et al., 2009; Alaska

Native Harbor Seal Commission Biosampling Program, Alaska Native Tribal Health Consortium, Division of Community Health, Community Environment and Safety Department; http://harborsealcommission.org/biosample.htm), and distribution of filter paper sample kits (including instructions and pre-paid shipping labels) through outlets like these would benefit mercury and other disease/health monitoring efforts around the globe (Curry, et al., 2011).

Our findings demonstrate that mercury in blood elutes readily, and our methods allow half of the eluate and roughly half of the mercury to remain with the post-elution filter paper (FP_E) (Fig. 4.4). We also show that Hg-associated dry components of blood likely distribute in a similar way by using a correction factor that demonstrated results with a strong correlation to WB [THg]. Since mercury is bound to sulfhydryl groups on hemoglobin molecules (Weed et al., 1962), we hypothesize that the hemoglobin is following this same pattern and is moving into the eluate, and half of that remains on the FP_E with the residual 200 µL of buffer. Based on this we have developed a conceptual model of the elution process describing the utility of predicting WB [THg] directly using blood soaked FP_{WB} and indirect methods that use certain post elution products (FP_E, E) (Fig. 4.4).

While blood is not as easy to collect as hair, filter paper technology facilitates blood collection and makes it easier to store and ship air-dried blood. Hair provides a long-term picture of mercury status (Budtz-Jorgensen et al., 2004), while blood represents short-term exposure, and is the route of exposure for target organs (the central nervous system and kidneys). The combination of dried filter papers and hair samples, both of which can be stored at room temperature and shipped under ambient conditions will allow wildlife scientists to obtain a more complete picture of the mercury status in populations of interest.

The designed use of these filter papers is for protein (antibody) preservation for antibody detection (serology). We have shown the added advantage of being able to use either FP_{WB}, FP_E, or E for quantifying mercury in whole blood. Previous studies have used filter paper eluate to validate serologic use in wildlife populations (Curry et al., 2011). We emphasize the excellent correlations between [THg] in WB and both FP_E and E (Fig. 4.4). Thus one can utilize the filter paper eluate (E) as intended for serology, and use any remaining FP_{WB} or FP_E to quantify mercury. This type of use could be a significant advantage if the available blood volume is limited, either in small species, or in situations where hunters or wildlife professionals are unwilling or unable to obtain large quantities of blood.

One unknown factor at this point pertains to the shelf life of these samples. All of our analyses were conducted within 8 months of collecting samples on filter papers. It would be important to see if similar results would be obtained with long-term storage. However, we do not anticipate volatilization or degradation to be significant for [THg] measures as compared to more vulnerable components such as antibodies.

In summary, Advantec Nobuto cellulose filter papers, by virtue of low background mercury (below detection), no signal interference, and uniform design, allow for reliable quantification of [THg] in whole blood. They are easy to transport, easy to use, and do not have to be refrigerated or frozen following sample collection and air-drying. Additionally, the filter papers may lend themselves to dual-purpose diagnostics via serology and [THg] quantification, which may be especially important in small species and under field conditions.

4.5 Conclusion

This filter paper technique promises to be broadly applicable wherever field sampling of whole blood for [THg] is needed. The strips can be air dried, do not need to be refrigerated, and theoretically have a long, stable shelf life once samples are collected. This method will be particularly useful in monitoring [THg] in subsistence foods in remote Alaskan communities, where Alaska Native peoples often subsist on fish eating marine mammals. Application of this technology to human fish consumer blood sampling, in conjunction with hair monitoring programs, should also be considered.

4.6 Acknowledgments

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Table 4.1: Mean, range, standard deviation (S.D.), and sample number (n) for total mercury concentration [THg] in bottlenose dolphin (*T. truncatus*, n=25) and harbor seal (*P. vitulina*, n=34) whole blood (WB), filter paper (FP_{WB}, FP_E), and eluate (E) samples.

Species	WB		FP_{WB}		FP_E		E	
	μg/g		μg/g		μg/g		μg/g	
	Wet*	Dry**	Wet**	Dry*	Wet**	Dry*	Wet*	CF Applied**
Bottlenose Dolphin								
Mean	0.48	2.39	0.41	2.06	0.39	1.97	0.12	0.50
S.D.	0.33	1.66	0.28	1.42	0.27	1.42	0.08	0.35
Range	0.12-1.34	0.61-6.71	0.09-1.15	0.47-5.75	0.09-0.97	0.45-5.67	0.03-0.35	0.13-1.51
Harbor Seal								
Mean	0.16	0.64	0.14	0.56	0.13	0.55	0.03	0.13
S.D.	0.11	0.42	0.10	0.38	0.09	0.36	0.02	0.09
Range	0.03-0.45	0.12-1.78	0.03-0.42	0.12-1.67	0.04-0.41	0.14-1.66	0.01-0.11	0.03-0.46

*Measured, **Calculated

FP_{WB}: FP soaked in whole blood, FP_E: Post-elution FP, E: Eluate

CF: Correction Factor

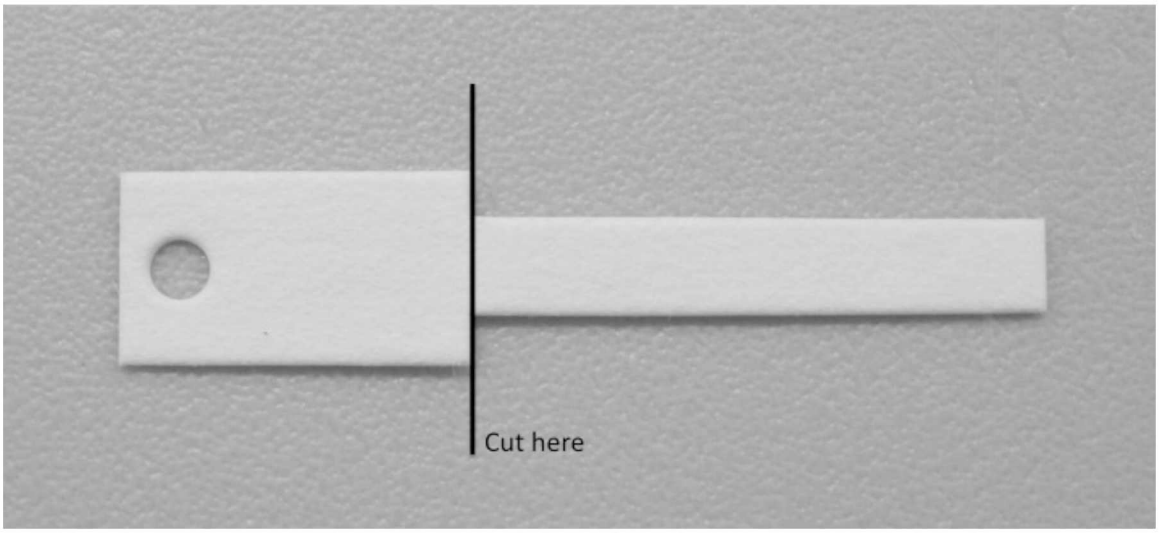

Figure 4.1: Single filter paper (FP) with placement of post blood soaking cut for processing marked with black line.

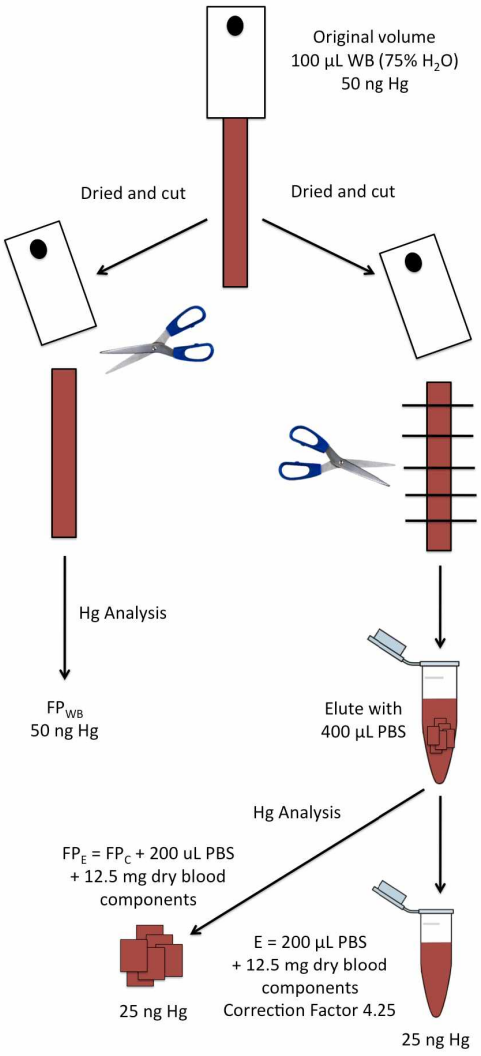

Figure 4.2: A conceptual model of the elution process. Each filter paper is soaked in approximately 100 µL of whole blood (original volume). After drying, approximately 20-25 mg (half) of dry blood products remain on filter paper (FP_{WB}). These FP_{WB} can be analyzed for [THg] directly, or eluted as follows. The dry blood products on FP_{WB} are eluted in 400 µL of PBS, 200 µL is collected as eluate (the remaining 200 µL remains soaked into FP_E). FP_E or E can then be analyzed for [THg].

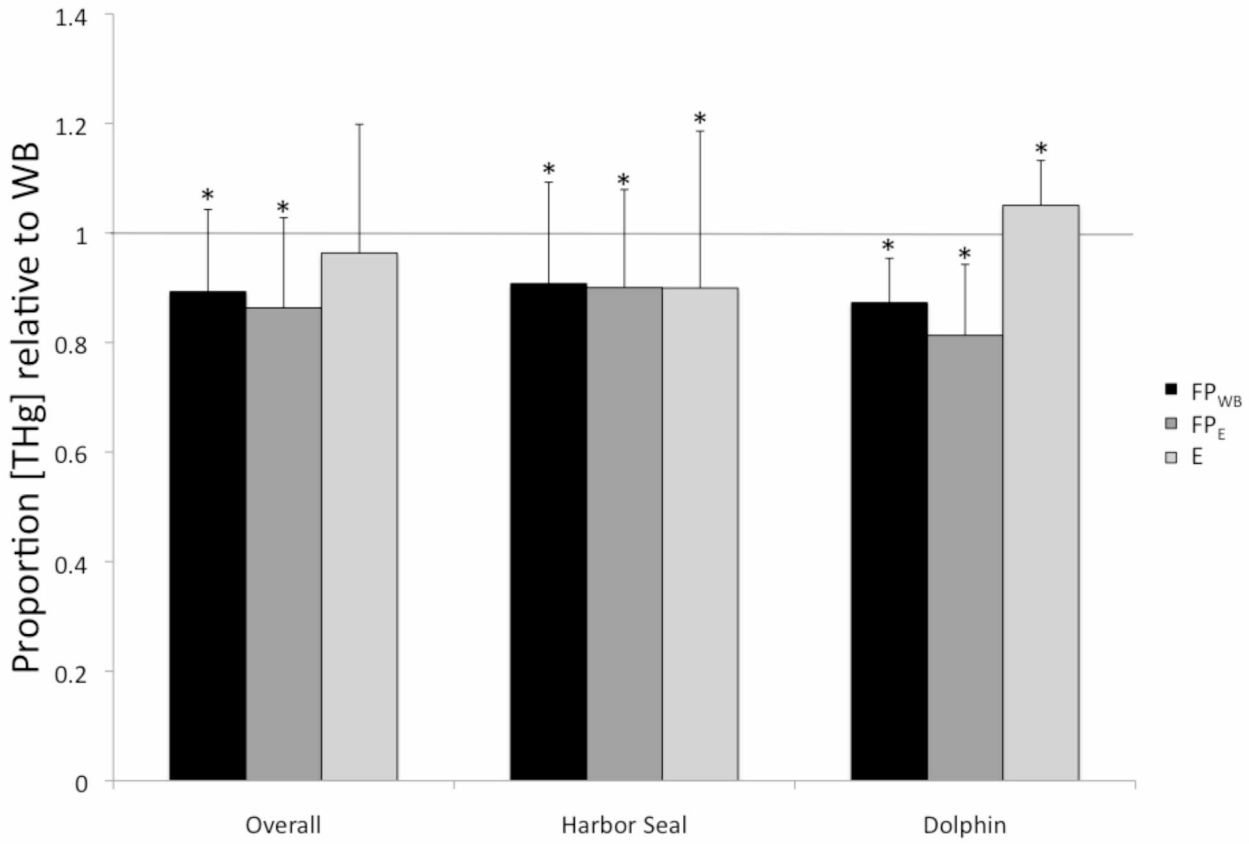

Figure 4.3: Proportion of [THg] (μg/g) in FP_{WB}, FP_E, and E relative to whole blood (WB = 1) for bottlenose dolphin (n=25) and harbor seal (n=34) samples. Error bars indicate one standard deviation from the mean. *Indicates significant difference in means of paired samples when compared to WB as gold standard ($p<0.05$).

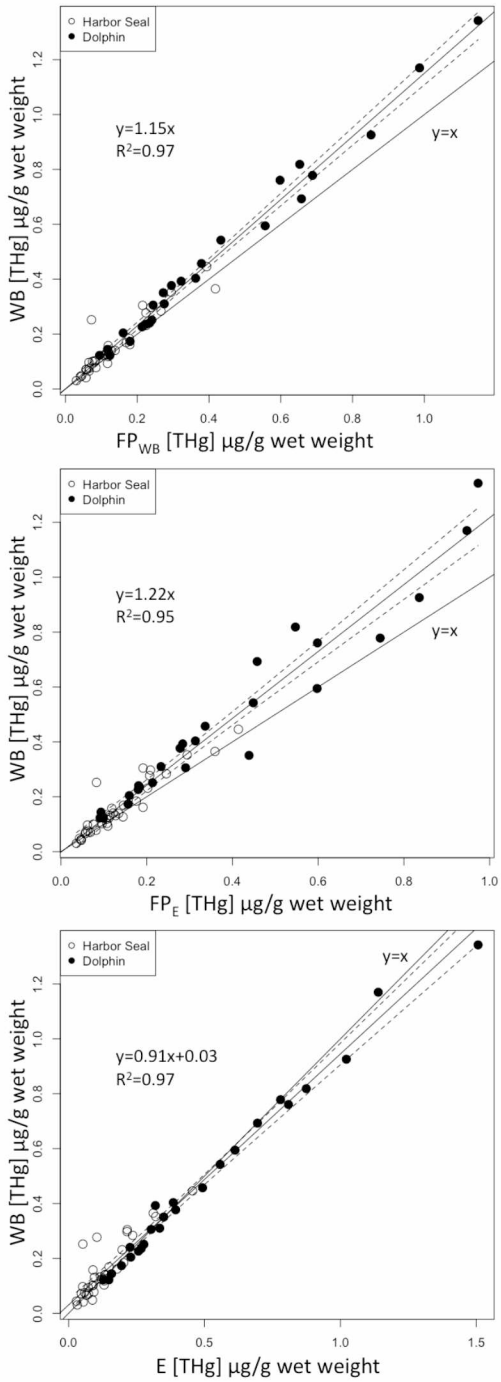

Figure 4.4: Linear regressions of WB on FP_{WB}, FP_E, and E (with dilution factor applied). All slopes are not equal to one. Dashed lines are a 95% confidence band for the slope. A line of unity (y=x) is shown in each panel.

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General Conclusions

The studies included in this thesis were aimed at developing and implementing new ways to survey for infectious and noninfectious disease agents in wildlife. Given the emergence of the One Health concept, that most infectious diseases in humans are of animal origin (1), and that a changing climate will modify the pattern of disease on the landscape (2), it is important to have reliable diagnostic methods ready. First, the history and epidemiology of tularemia, a zoonotic disease of importance to the state of Alaska (and the Northern hemisphere) was reviewed for the state of Alaska (3). Then a broad-based 16S ribosomal RNA gene PCR and sequencing technique was developed and implemented by screening a wide variety of wildlife tissue samples. Next, this broad-based 16S rRNA gene PCR technique was used as an initial screening and identification tool in an investigation of avian embryo mortality on the Arctic Coastal Plain of Alaska. Finally, a filter paper-based method for quantifying whole blood mercury levels was validated and shown to be a potentially very useful field sampling technique (4).

I began by reviewing tularemia in Alaska, a zoonotic disease that is present in the state of Alaska and throughout the Northern Hemisphere. Epidemiologic work shows that most human tularemia patients who reported recent animal exposure stated exposure to hare or muskrat, which fits with either or both of them being a suspected reservoir (3). Molecular typing of recent Alaskan *F. tularensis* isolates suggest either multiple introductions of *F. tularensis* to the state, or that *F. tularensis* has been present in the state for a long time and has diversified greatly. Further work is warranted to increase our understanding of tularemia in Alaska and will help guide future public health surveillance and intervention.

Accurate and broad-based methods are needed to identify bacterial pathogens (such as *Francisella tularensis*) in animal tissue samples. The next chapter of this thesis described the development of a PCR method and a qPCR method for identifying bacterial DNA in tissues. The methods that we developed work particularly well in animals where infectious disease is suspected (i.e. necropsy specimens), vs. as a screening tool for large populations. In many cases interpretable DNA sequences are obtained without the need for cloning.

The major advantages to the PCR techniques described here is that they are extremely broad-based (i.e. not agent specific). Additionally, these methods are cost effective, quick, and the qPCR protocol leaves potential for high-throughput. Limitations to these techniques include that it can only be used on tissues from sterile areas of the body. Also, either of the PCR methods developed here will amplify pathogen DNA, but will also amplify DNA from non-pathogenic and contaminant organisms, so care must be taken when interpreting sequence results. Despite these limitations, these broad-based PCR methods have a place in the identification of bacterial disease in wildlife.

Following the development of broad-based PCR surveillance methods for bacterial DNA, we were able to use these methods to investigate causes of embryo mortality in greater white-fronted geese (*Anser albifrons*) in Arctic Alaska. We identified 11 species of bacteria in the contents of nonviable (addled) white-fronted goose eggs, and showed that the 4 most commonly isolated bacteria are capable of causing embryo mortality by performing an infection study.

Surprisingly, we identified a bacterium in the genus *Neisseria* in the majority of our addled eggs. We are unaware of any reports of species in this genus being isolated from bird eggs. Based on DNA sequence data, our *Neisseria* isolate is most closely related to *N.*

canis or *N. animaloris*, but none share more than 97% 16S rRNA gene sequence identity. Additional sequencing of the cpn60 gene hints that this is a unique bacterium in the genus. We do not know the source or route of infection, and swab data reveal that *Neisseria* DNA is present in nest material, on eggshells, and in the cloacae of female geese. Future work should focus on identifying the source and route of transmission of these bacteria.

Following this investigation of avian embryo mortality, we focused our efforts on developing a filter paper-based method for monitoring mercury exposure in whole blood in wild animals. These data show that filter paper samples are an accurate and reproducible way to quantify whole blood total mercury [THg] for some animals (4).

Overall recoveries on a concentration basis were excellent, and our R^2 values show that whole blood [THg] can be estimated from dried or eluted filter paper samples. The findings from this chapter show that the mercury in blood elutes readily from the filter papers. We showed that roughly half of the eluate remains with the post-elution filter paper, and that Hg-associated dry components of blood likely distribute in a similar way. From this, we developed a conceptual model of the elution process that shows where the mercury in an initial whole blood sample ends up whether you analyze a whole air-dried FP or elute and analyze [THg] on an eluted sample or post-elution FP.

This thesis as a whole has investigated the use of two novel methods for surveillance of infectious and noninfectious disease in wildlife. We also used one of these techniques in the first investigation into avian embryo mortality in waterfowl and in the Arctic. These methods promise to be valuable tools for wildlife professionals and will benefit human, wildlife, and ecosystem health.

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